Apoptin

Oncogenic Transformation & Tumor-selective Apoptosis

Rhyenne M.E. Zimmerman

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Promotor

Prof. dr. M.H.M. Noteborn

Co-promotor

Dr. C. Backendorf

Overige leden

Prof. dr. J.P. Abrahams

Prof. dr. J. Brouwer

Prof. dr. H.P. Spaink

Prof. dr. J. Sun (Huazhong University, Wuhan, China)

Dr. Y.H. Zhang

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Dedicated to Mary and the Holy Trinity,

in loving memory of those who taught me about Love and Faith

Si kaka ta tin wesu, awe l'e la lanta balia rumba riba mesa

Table of contents

Chapter 1

Thesis outline

Cancer is one of the leading causes of death worldwide. Treatment is hampered by an incomplete understanding of the mechanisms underlying carcinogenesis and, consequently, by the absence of therapies to specifically eradicate cancer cells without harming normal, healthy cells. Intriguingly, the avian-virus derived protein apoptin was found to selectively induce apoptosis in transformed and tumor cells, heralding the advent of a new era in cancer treatment.

The aim of this thesis was to discover the path followed by apoptin to distinguish between normal and cancer cells, and selectively kill the latter, in order to a) get to the root of the problem that is cancer, and b) provide the knowledge which is necessary to design novel, more selective, more effective, safe anti-tumor therapies. To this end, we identified a number of apoptin-interacting proteins, and studied their roles in tumor-selective apoptin-induced apoptosis.

Chapter 2 summarizes current knowledge on normal regulation of cellular proliferation and the derailments thereof leading to malignant transformation, as well as novel strategies in cancer treatment. Since the discovery of apoptin, a number of other cellular and viral proteins have also been shown to induce tumor-selective cytotoxicity; in **chapter 3**, an overview is presented of apoptin, and these other proteins killing tumor cells (PKTC).

Chapter 4 introduces a novel apoptin-interacting protein, FAM96B. Functional analysis implicates FAM96B in the regulation of the cell cycle, including the processes of sensing DNA damage and establishing sister chromatid cohesion. In **chapter 5**, apoptin's activities in the tumor cell nucleus are investigated, and chromatin-bound apoptin is found to associate with various nucleolar proteins that are involved in the regulation of ribosome biogenesis, the DNA damage response and cell cycle regulation. The data suggest that apoptin coordinates tumor-selective apoptosis at least partially from

within the nucleolus. **Chapter 6** analyzes the roles of the apoptin-interacting breast cancer associated protein BCA3 and that of the major tumor suppressor protein phosphatase 2A (PP2A) in the phosphorylation of apoptin.

Finally, the data are compiled in **chapter 7**, where novel insights into the cancer blueprint, the path taken by apoptin to sense it and effectuate cancer cell death, as well as the relevance for the design of future cancer therapies are discussed.

Chapter 2

Introduction

Cellular proliferation and oncogenic transformation: uncovering the fundamental principles for specific killing of cancer cells

Abstract

The *Book of Genesis* gives a detailed account of how God created our planet in 7 days - or, rather, 6 - through a set of specific, sequential actions. In his *On the Origin of Species*, Charles Darwin postulated that all species of life emerged from a limited number of common ancestors, evolving over time through natural selection (Darwin, 1859). However large the contradiction, both books served an identical purpose: to explain the origin of life. So too in medicine, it was believed that illnesses were the result of supernatural or divine forces, until Hippocrates first argued that disease was the product of environmental factors, diet, and living habits (Jones, 1868). Although many of his assumptions turned out to be erroneous, the so-called 'father of medicine' did launch the idea of pathogenesis, a concept fundamental to modern life science research. Combining the insights of Hippocrates and Darwin, and of many of their colleagues in-between and since, intense scientific effort has been directed at understanding the pathogenesis of one of the world's largest contemporary health problems: cancer (WHO, 2008). While the elaborate molecular mechanisms behind tumorigenesis are being elucidated more and more clearly, therapy is still lacking in safety and effectiveness. Here, I will review the current knowledge on carcinogenic cell transformation, as well as therapeutic approaches stemming from these findings. Next, I will describe exciting new prospects in both research and therapy, where, finally, I will highlight the anti-cancer potential of the Chicken Anemia Virus-derived protein apoptin.

2.1 In the beginning, there was chaos – on the origin of cancer

Cancer is the general term for a class of diseases, characterized by uncontrolled cellular proliferation. Research has indicated that cancer development (tumorigenesis) originates with the stepwise accumulation of genetic changes, driving the progressive transformation of normal cells into highly malignant progeny (Hahn and Weinberg, 2002). These genetic changes include mutations, deletions and amplifications, producing oncogenes with dominant gain of function, and tumor suppressor genes with recessive loss of function. The vast majority of all known tumor suppressor genes are involved in DNA repair and genomic regulation (Lengauer, et al., 1998), so that tumor cells almost invariably display a large degree of genomic instability, resulting in further accumulation of malignant genetic changes.

Random mutations in the approximately six billion basepairs comprising the human genome could theoretically give rise to a huge number of different combinations of genetic alterations. However, research indicates that the process of carcinogenesis is not a random one, and it has been suggested that the more than 100 different types of human cancer share at least six crucial characteristics, the so-called core 'hallmarks' of cancer (Hanahan and Weinberg, 2000, 2011; Stratton, et al., 2009):

1. self-sufficiency in growth signals
2. insensitivity to growth-inhibitory signals
3. evasion of programmed cell death
4. limitless replicative potential
5. sustained angiogenesis
6. tissue invasion and metastasis

Researchers now also propose two additional alterations, namely a change in cellular metabolism (Weinberg and Chandel, 2009), and evasion of immune destruction (Hanahan and Weinberg, 2011). As will

be discussed in following sections, each of these acquired capabilities represents the breach of regulatory mechanisms tightly controlling the cell cycle and hence normal proliferation and homeostasis, upsetting the balance between cell survival and proliferation, and cell death. The genomic instability discussed above is regarded as an enabling characteristic, as is the tumor micro-environment, which can secrete growth and inflammatory factors to promote neoplastic progression (see below).

2.2 Normal proliferation and homeostasis: the cell cycle

At the basis of cellular proliferation and homeostasis lies the cell cycle. This set of strictly organized processes dictates if, when and under which conditions a cell reproduces itself, and provides safe-guarding mechanisms to dispose of aberrant cells.

The most fundamental function of the cell cycle is to accurately duplicate the cell's chromosomal DNA and then segregate the copies precisely into two genetically identical daughter cells. These processes define the two major phases of the cell cycle (Figure 2.1) (Heichman and Roberts, 1994). DNA duplication occurs during S phase (S for synthesis), and chromosome segregation and cell division occur in M phase (M for mitosis). Before each of these phases, eukaryotic cells go through a so-called 'gap' phase – G1 between M and S phase, and G2 between S and M phase. This is partly to allow time for growth, but also importantly to provide time for the cell to monitor the internal and external environment, ensuring that conditions are suitable and all preparations have been completed. The G1 phase is especially important in this respect. Its length can vary greatly depending on external conditions and extracellular signals from other cells. If extracellular conditions are unfavorable, for example, cells delay progress through G1 and may even enter a specialized resting state known as quiescence, or G0, in which they can remain for days, weeks, or even years before resuming proliferation (Pardee, 1989). In

fact, many cells remain permanently in G0 until they or the organism dies. Such cells have either differentiated into specialized states, or have become senescent, and do not have the ability to return to G1. Typically, cells in G2 that do not meet the requirements for completion of the cell cycle, e.g. because of extensive DNA damage, are killed. This is achieved through various modes of cell death (see section 2.5.1).

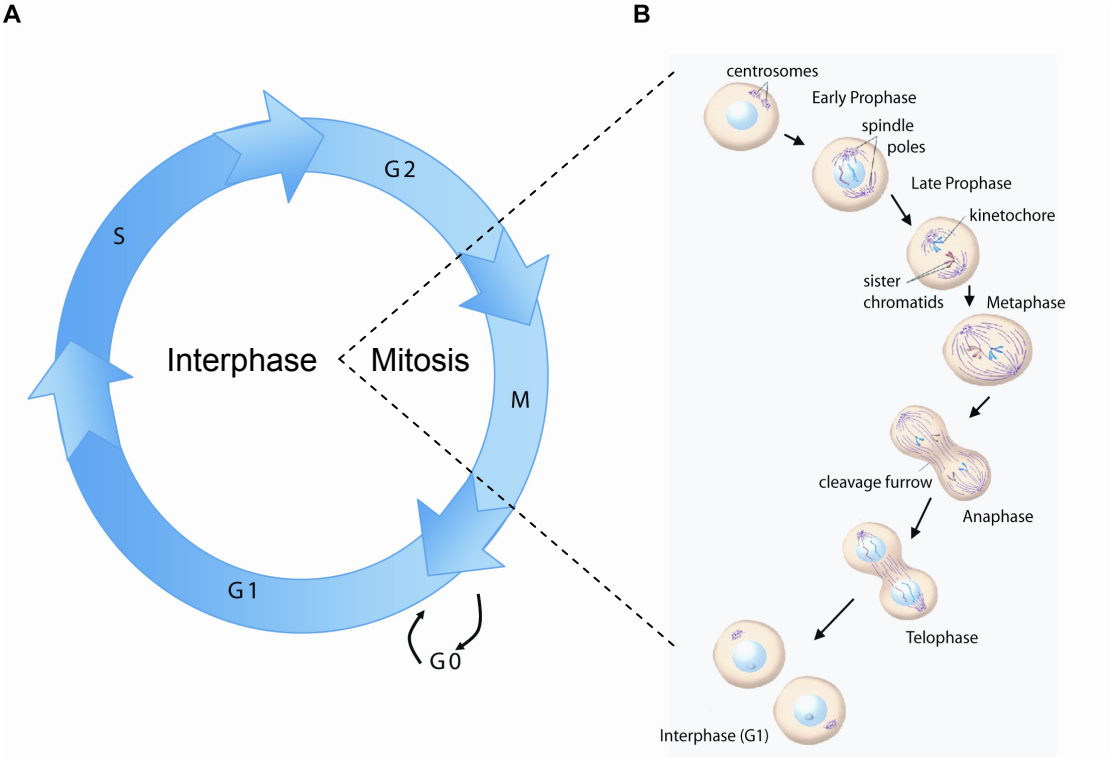

Figure 2.1. A. The eukaryotic cell cycle is traditionally divided into four sequential phases: G1, S, G2, and M. G1, S, and G2 together are called interphase. **B.** During interphase, the centrioles are also replicated, forming small daughter centrioles. Early prophase: the centrosomes, each with a daughter centriole, begin moving toward opposite poles of the cell. Chromosome condensation and nuclear membrane disintegration are initiated. Late prophase: chromosome condensation is completed; each visible chromosome structure is composed of two chromatids held together at their centromeres. The microtubular spindle fibers begin to radiate from the regions just adjacent to the centrosomes, which are moving closer to their poles. Some spindle fibers reach from pole to pole; most go to chromatids and attach at kinetochores. Metaphase: the chromosomes move toward the equator of the cell, where they become aligned in the equatorial plane. Anaphase: the two sister chromatids separate into independent chromosomes and move to one spindle pole each. Simultaneously, the cell elongates, and cytokinesis begins as the cleavage furrow starts to form. Telophase: new nuclear membranes form around the daughter nuclei; the chromosomes uncoil and become decondensed; and the nucleolus becomes visible again. Cytokinesis is nearly complete, and the spindle disappears as the microtubules and other fibers depolymerize. Upon the completion of cytokinesis, each daughter cell enters the G1 phase of the cell cycle and is ready to proceed again around the cycle. Adapted from Lodish et al. (1999)

Box 1. *Cyclins and CDKs control the cell cycle*
At the heart of the cell-cycle control system is a family of protein kinases known as cyclin-dependent kinases (Cdks), which are sequentially activated to trigger the various steps of the cell cycle (Norbury and Nurse, 1991, 1992). Cdks are activated by the binding of cyclins – as indicated by their name – as well as by phosphorylation and dephosphorylation of the kinase. They are inactivated by various Cdk inhibitory proteins (CKIs), such as p16Ink4a, p27Kip1, and p21Cip1, and by degradation of the cyclin subunits at specific stages of the cell cycle (Elledge and Harper, 1994). Each cyclin is specific for a given phase of the cell cycle, and the levels of the various cyclins rise and fall as the cell progresses through the cycle. This results directly in cyclical changes in the phosphorylation and (in)activation of intracellular proteins that initiate or regulate the major events of the cell cycle: DNA replication, mitosis, and cytokinesis. The major cell-cycle regulatory proteins are summarized in Table 2-1.

Table 2-1. Specific Cyclin-Cdk complexes act to promote each phase of the cell cycle.

Cell cycle phase	**Cyclin**	**Cdk**
G1	Cyclin D	Cdk4/6
G1/S	Cyclin E	Cdk2
S	Cyclin A	Cdk2
M	Cyclin B	Cdk1

Below, the four phases of the cell cycle are discussed in further detail.

2.2.1 G1

During the G1 phase of the cell cycle, cells respond to extracellular signals by either advancing toward another division or withdrawing from the cycle into G0 (Sherr, 1996). G1 progression normally relies on stimulation by mitogens, e.g. Ras, and can be blocked by anti-proliferative cytokines, e.g. TNFβ.

Early in G1, D-type cyclins (see Box 1) assemble into holoenzyme complexes with one of two catalytic subunits, Cdk4 or Cdk6 (Sherr, 1994). Transcription of the cyclin D1 gene and assembly with Cdk4 depend strongly on receptor-mediated Ras and PI3-K signaling (Figure

2.2A) (Marshall, 1999). Persistent mitogenic stimulation leads to progressive accumulation of cyclin D-dependent kinases within the cell nucleus; here they collaborate with cyclin E-Cdk2 to phosphorylate pRb and pRb family members p107 and p130, canceling their growth inhibitory functions by disrupting the interaction with E2F, resulting in activation of G1/S and S-phase cyclins, thereby activating the DNA replication machinery and facilitating S phase entry (Reed, 1992).

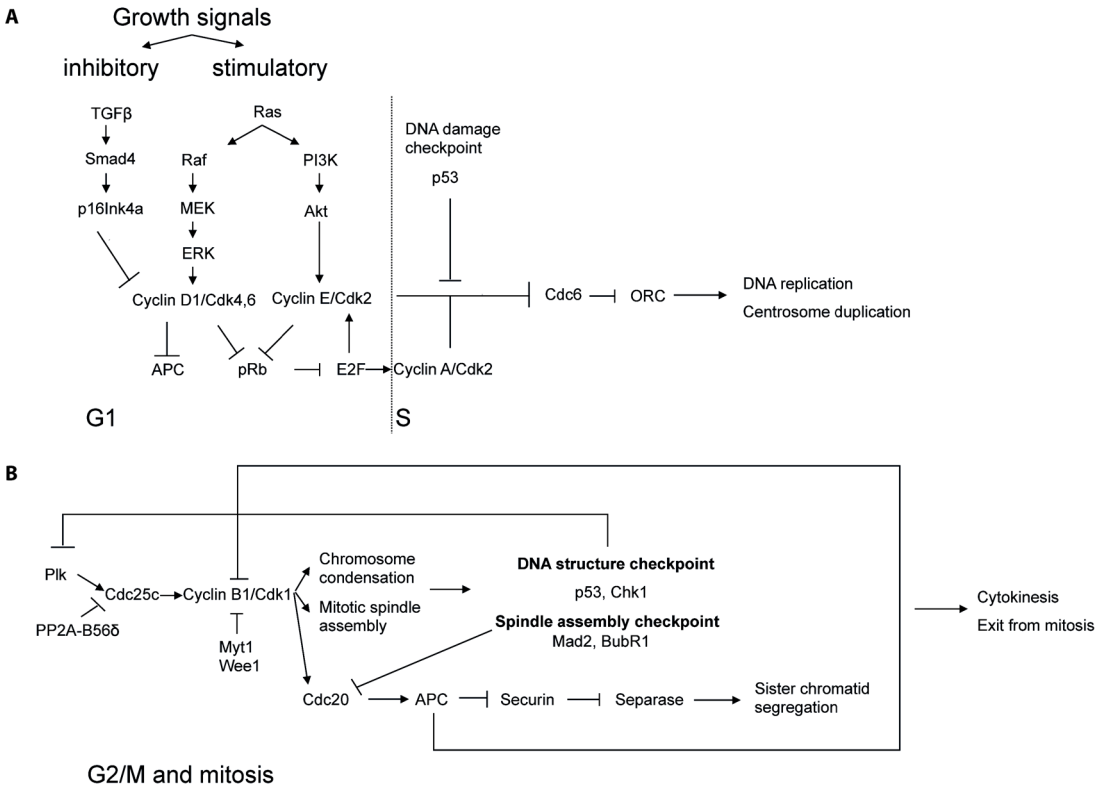

Figure 2.2. Molecular pathways comprising the four phases of the cell cycle. **A.** In G1, growth stimulatory such as Ras, and growth inhibitory signals such as TGFβ, converge on the cyclinD1/Cdk4 complex. A net balance of positive signals lead to activation of cyclinD1/Cdk4, which cooperates with cyclinE/Cdk2 to phosphorylate pRb, thus liberating E2F and initiating DNA replication. ORC, origin recognition complex. **B.** Following DNA replication, CyclinB1/Cdk1 is activated through the actions of Polo like kinase. This activity is however subject to two G2/M control checkpoints, namely the DNA structure checkpoint, which ensures the absence of unreplicated or damaged DNA, and the spindle assembly checkpoint, which ensures the attachment of all sister chromatids to microtubules connecting them to opposite poles of the spindle. Successful clearance of these checkpoints results in activation of the APC, which results in sister chromatid separation and completion of cell division. See text for further details.

The phosphorylation and thus inactivation of pRb constitutes a so-called restriction point (Blomen and Boonstra, 2007; Pardee, 1974); after this, the cells become refractory to extracellular growth regulatory signals, and are committed to enter S phase and complete the cell cycle. Beyond this point, the cell cycle can only be halted by activation of the cell cycle checkpoints (see Box 2).

Box 2. *The G1/S cell cycle checkpoint*
Although cell cycle transitions depend on the underlying CDK cycle, superimposed checkpoint controls help ensure that certain processes are completed before others begin. Components of checkpoint control need not be essential to the workings of the cycle; instead, their role is to brake the cycle in the face of stress or damage. By allowing repair to take place, they become crucial in maintaining genomic stability (Sancar, et al., 2004).

At the transition from G1 to S, there is an important such checkpoint: if the cell's DNA is damaged, p53 (along with its family members p63 and p73) is activated (Bartek, 2001). One of its roles is to ensure that, in response to genotoxic damage, cells arrest in G1 and attempt to repair their DNA before it is replicated. If the damage is too severe to be repaired, continued activation of p53 leads to programmed cell death (see section 2.5.1). If however, the damage is repaired, p53 is again inactivated, and the cell continues through to S phase.

2.2.2 S phase

S phase begins with the activation of the pre-replication complexes by cyclin A/E-Cdk2 (Wuarin and Nurse, 1996). The DNA pre-replication complexes are assembled on replication origins during G1, and are kept inactive by the binding of Cdc6. Phosphorylation of Cdc6 by S-phase Cdk complexes not only activates initiation of DNA replication but also prevents re-assembly of new pre-replication complexes. Because of this inhibition, each chromosome is replicated just once during passage through the cell cycle, ensuring that the proper chromosome number is maintained in the daughter cells.

2.2.3 G2

At the end of S-phase, before progression to M-phase, there are two checkpoints (Sancar, et al., 2004): one in early G2, to ensure all DNA has been replicated, and one in late G2, ensuring that the replicated DNA is error-free. If both checkpoints are cleared successfully, Polo-like kinase activates Cdc25c, which itself activates cyclinB/Cdk1 by removing the inhibitory phosphorylations catalyzed by the Myt1 and Wee1 kinases.

2.2.4 Mitosis

Following its activation by Cdc25c, the cyclinB/Cdk1 complex triggers chromosome condensation, assembly of the mitotic spindle, nuclear envelope breakdown, and rearrangement of the actin cytoskeleton, Golgi apparatus, and ER (Figure 2.2B) (Colanzi and Corda, 2007; Güttinger, et al., 2009). At the metaphase-to-anaphase transition, there is a final, major checkpoint: the spindle-attachment checkpoint (Musacchio and Salmon, 2007). At this point, the cell contains 4n DNA, with each replicated chromosome consisting of two identical sister chromatids glued together along their length by the action of protein complexes called cohesins. The two sister chromatids are attached to opposite poles of the mitotic spindle, with cohesion being enforced by the action of securin. Upon the initiation of anaphase, Cdc20 activates the anaphase promoting complex (APC), which then targets securin for proteolysis, freeing separase, which itself cleaves the cohesin complexes, allowing segregation of the sister chromatids (Sullivan and Morgan, 2007).

The spindle-assembly checkpoint (SAC) operates to ensure that all chromosomes are properly attached to the spindle before sister-chromatid segregation occurs. The SAC depends on a sensor mechanism that monitors the state of the kinetochore, the specialized region of the chromosome that attaches to microtubules of the spindle. The kinetochore comprises the chromosome centromere,

which is defined by the incorporation of specific histone variants, including CENP-A (Cleveland, et al., 2003), and achievement of proper kinetochore tension is dependent on proper formation of pericentric heterochromatin, which is characterized by trimethylation of histone H3 lysine 9 and H4 lysine 20 (Heit, et al., 2009). The generation of stable kinetochore-microtubule attachments depends on the B56 regulatory subunit-containing protein phosphatase PP2A, which is enriched at centromeres/kinetochores of unattached chromosomes (Foley, et al., 2011).

Any kinetochore that is not properly attached to the spindle sends out a negative signal to the cell-cycle control system, blocking Cdc20-APC activation and sister-chromatid segregation. The nature of the signal generated by an unattached kinetochore is not clear, although several proteins, including Mad2, are recruited to unattached kinetochores and are required for the SAC to function. Even a single unattached kinetochore in the cell results in Mad2 binding and the inhibition of Cdc20-APC activity and securin destruction. Furthermore, proteins such as BubR1 sense kinetochore tension, activating the SAC upon lack of proper, amphitelic (bi-oriented) attachment of sister chromatids. Thus, sister-chromatid segregation cannot occur until the final kinetochore has been attached, and sister chromatids are attached to opposite poles of the spindle.

After the chromosomes have segregated to the spindle poles, the cell must reverse the complex changes of early mitosis. The spindle must be disassembled, the chromosomes decondensed, and the nuclear envelope reformed. Cytokinesis then ensues, the cytoplasm is pinched off, and two identical daughter cells are produced, completing the cell cycle. The exit from mitosis is triggered by the inactivation of cyclinB/Cdk1 (Wolf, et al., 2007). This inactivation occurs mainly by ubiquitin-dependent proteolysis of cyclin B, triggered by the same Cdc20-APC complex that promotes the destruction of securin at the

metaphase-to-anaphase transition. Thus, the activation of the Cdc20-APC complex leads not only to anaphase, but also to inactivation of the cyclin B/Cdk1 complex — which in turn leads to all of the other events that take the cell out of mitosis.

Recent studies have shown that the cyclin B/Cdk1 complex can also be inactivated by phosphorylation and inactivation of Cdk1, providing an important contribution to the exit from mitosis. Phosphorylation of Cdk1 is achieved by inactivation of Cdc25c, which again is achieved through the activities of PP2A, specifically PP2A complexes containing the B56δ subunit (Forester, et al., 2007).

2.3 Mechanisms underlying uncontrolled proliferation in cancer: hallmarks and enabling characteristics

As indicated before, human cancer cells have acquired certain capabilities, which allow them to breach the regulatory mechanisms of the normal cell cycle, conferring upon themselves the aforementioned trademark characteristics. Each trait is described below, with a few examples illustrating the strategies by which they are acquired in human cancers.

Self-sufficiency in proliferative signaling

Oncogenic processes exert their greatest effect by targeting particular regulators of G1 phase progression. Cancer cells commonly achieve autonomy from normal growth signaling through three molecular strategies, involving alteration of:

- Extracellular growth signals: many cancer cells acquire the ability to synthesize the growth factors to which they are responsive, e.g. PDGF (Ostman and Heldin, 2007; Wang, et al., 2010), EGF and TGFα (Kalyankrishna and Grandis, 2006). Alternatively, cancer cells may send signals to stimulate the release of growth factors by surrounding (normal) stromal cells (Bhowmick, et al., 2004; Cheng, et al., 2008).

- Transcellular transducers of those signals: growth factor receptors are often overexpressed or structurally altered in many cancers, e.g. Her2/neu in breast cancer (Freudenberg, et al., 2009), either allowing cells to become hyperresponsive to ambient levels of growth factors that normally would not trigger proliferation, or eliciting ligand-independent signaling, respectively.
- Intracellular circuits that translate those signals into action: e.g. the B-Raf protein is activated in about 40% of human melanomas, continuously stimulating proliferation. Similarly, activating mutations in the catalytic subunit of PI3K are being detected in an array of tumor types (Jiang and Liu, 2009; Yuan and Cantley, 2008).

Recent results have also highlighted the importance of the disruption of negative-feedback loops in cancer cells. In approximately 20% of human tumors, the Ras oncogene is activated (Davies, 2002; Downward, 2003; Karnoub and Weinberg, 2008). However, its oncogenic effects do not result from a concomitant hyperactivation of its downstream signaling pathways. Instead, Ras GTPase activity, which normally operates as an intrinsic negative-feedback mechanism to ensure that active signaling is transitory, is compromised.

Circumventing growth-inhibitory signaling

As discussed in paragraph 2.2.1, up to the restriction point, progression through the cell cycle is controlled by the effects of extracellular signals on pRb; beyond this point, control is executed via the cell cycle checkpoints. Hence, to achieve insensitivity to inhibitory signaling, cells must disable the TGFβ-pRb pathway, as well as the cell cycle checkpoints.

Disruption of the TGFβ-pRb signaling circuit, thereby acquiring insensitivity to anti-growth signals (Massagué, 2004), can be achieved in a number of ways:

- downregulation or mutation of the TGF-β receptors (Levy and Hill, 2006);
- elimination of intracellular signal transducers, e.g. by mutation of the gene encoding for Smad4 (Levy and Hill, 2006);
- loss of functional pRb; in fact, the pRb gene was the first tumor suppressor gene to be identified (Knudson, 1971; Sherr and McCormick, 2002).

The first and most important cell-cycle checkpoint (Box 2) involves the activation of another major tumor suppressor protein, p53. Whereas pRb acts in response to signals from the outside, p53 responds to signals from within the cell. If there is significant damage to the cell's genome, or if the levels of growth-promoting signals, nucleotide pools, glucose, or oxygenation are suboptimal, p53 can halt further cell-cycle progression until these conditions have normalized, or, in the face of overwhelming or irreparable damage to such cellular subsystems, p53 may trigger apoptosis. Accordingly, p53 function is lost in over 50% of human tumors, either directly as a result of mutations in the p53 gene, or indirectly through binding to (viral) proteins, or as a result of alterations in genes whose products interact with p53 or transmit information to or from p53 (Vogelstein, et al., 2000).

Evasion of cell death

The normal cell possesses the ability to detect cellular stress, including abnormal mitogenic stimulation, and responds by preventing further division through either cell cycle arrest or programmed cell death (see section 2.5.1), preventing the survival and proliferation of cells with various disease-promoting mutations. Though the exact mechanisms underlying this 'sensing' ability remain to be fully elucidated, several key players have been identified.

For example, excessive mitogenic stimulation leads to the production of a cell-cycle inhibitor protein called p14ARF, which binds and inhibits the p53-inhibitor Mdm2, therefore causing p53 levels to increase, inducing either cell-cycle arrest or, if prolonged, apoptotic cell death (Sherr, 2001). As discussed before, p53 is also activated in response to DNA damage. Furthermore, insufficient survival factor signaling can also trigger apoptosis (section 2.5.1).

Cancer cells acquire resistance to apoptosis through various mechanisms:

- the p53 tumor suppressor gene is inactivated by mutation in approximately half of all human cancers (Brosh and Rotter, 2009; Sherr and McCormick, 2002);
- the anti-apoptotic Bcl-2 oncogene is often up-regulated (Reed, 2008);
- the Fas death-inducing signal has been shown to be titrated away from the Fas death receptor by upregulation of a non-functional (decoy) Fas ligand in cancer cell lines (Pitti, et al., 1998).

Besides apoptosis, emerging evidence suggests that still other devices are in place to prevent abnormal cellular proliferation. These include autophagy, necrosis and senescence. However, it also seems that tumor cells might actively engage in these processes in order to achieve survival. Each pathway is discussed in detail in paragraph 2.5.1, though senescence will also be discussed in the next section.

Acquiring limitless replicative potential

In principle, the combination of growth signal autonomy, insensitivity to anti-growth signals and resistance to apoptosis should suffice to enable the generation of the vast cell mass constituting a tumor. However, Hayflick showed that cells in culture have a finite replication potential and stop growing after a certain number of doublings (60-70

for normal human cells) – a process termed senescence (Hayflick, 1965; Hayflick and Moorhead, 1961). Others showed that senescence could be circumvented by disabling the p53 and pRb tumor suppressor proteins, after which cells continue to multiply until they enter a second state, labeled crisis, which is characterized by massive cell death and end-to-end fusion of chromosomes (Hara, et al., 1991; Shay, et al., 1991).

It is this latter trait that provided the clue to cellular immortalization. The ends of chromosomes, telomeres, are progressively shortened with each cycle of cell division, due to the inability of DNA polymerases to completely replicate the 3' ends of the linear chromosomal DNA during S phase (Harley, et al., 1990; Zhao, et al., 2009). Once telomeres are shortened beyond a critical length, the protein complexes capping the ends are lost, and they are no longer able to protect the ends of chromosomal DNA. The unprotected chromosomal ends trigger a widespread DNA damage response, resulting in end-to-end fusions and death of the cell (Blackburn, 2000; d'Adda di Fagagna, et al., 2003).

In order to prevent telomere shortening and achieve immortalization, malignant cells must therefore activate a system for telomere maintenance (Samassekou, et al., 2010). The large majority (85-90%) does so by upregulating the expression of the telomerase enzyme (Counter, et al., 1994; Kim, et al., 1994; Shay and Bacchetti, 1997), which elongates telomeric DNA, while the remainder uses a mechanism termed "alternative lengthening of telomeres" (ALT), which appears to maintain telomeres through recombination-based interchromosomal exchanges (Bryan, et al., 1997, 1998; Morrish and Greider, 2009).

Angiogenesis

In order to attain and sustain their rapid proliferation rate, tumor cells need to generate an ample amount of ATP for energy and *de novo* synthesis of nucleotides, lipids and proteins. This results on the one hand in an increased demand for oxygen cq vasculature, and on the other hand a fundamental switch in cellular metabolism (the 'seventh' hallmark, see below). The oxygen and nutrients supplied by the vasculature are crucial for cell function and survival, obligating virtually all cells in a tissue to reside within 100 μm of a capillary blood vessel. In order to progress to a larger size, tumors must therefore develop angiogenic ability (Bergers and Benjamin, 2003). This "angiogenic switch" is activated by changing the balance of angiogenesis inducers and countervailing inhibitors. One common strategy involves increased expression of vascular endothelial growth factor (VEGF) (Cook and Figg, 2010); VEGF gene expression can be up-regulated by both hypoxia and oncogene signaling (Carmeliet, 2005; Ferrara, 2009; Mac Gabhann and Popel, 2008). Surprisingly, in both animal and human models, angiogenesis was found to be induced relatively early during the development of invasive cancers. It is therefore likely that the angiogenesis switch also contributes to the premalignant phase of neoplastic progression.

Tissue invasion and metastasis

In reality, the vast majority of human cancer deaths are not caused by the primary tumor, but rather by the metastases arising from it. Successful invasion and metastasis depend on the other hallmark acquired capabilities, as well as on the loss of adherence with the surrounding tissue. The most widely observed alteration in cell-cell adhesion in cancer involves E-cadherin (Berx and van Roy, 2009). Normally, coupling of adjacent cells by E-cadherin bridges results in the transmission of anti-growth and other signals via cytoplasmic contacts with beta-catenin to intracellular signaling circuits. Such "contact inhibition" is further enhanced by the actions of e.g. Merlin,

and LKB1. However, in the majority of epithelial cancers E-cadherin function is lost (e.g. by promoter hypermethylation), freeing the path to metastasis (Lombaerts, et al., 2006). Though it remains to be seen how frequently Merlin is compromised in human cancers, it is already known that the loss of the *NF2* gene, which encodes Merlin, triggers a form of human neurofibromatosis. Similarly *LKB1* has been identified as a tumor suppressor gene that is lost in certain human malignancies (Shaw, 2009), and suppression of LKB1 expression destabilizes epithelial integrity and renders epithelial cells susceptible to Myc-induced transformation (Hezel and Bardeesy, 2008; Partanen, et al., 2009).

The multistep process of invasion and metastasis has been schematized as a sequence of discrete steps, often termed the invasion-metastasis cascade (Talmadge and Fidler, 2010). This depiction envisions a succession of cell-biologic changes, beginning with local invasion, then intravasation by cancer cells into nearby blood and lymphatic vessels, transit of cancer cells through the lymphatic and hematogenous systems, followed by escape of cancer cells from the lumina of these vessels into the parenchyma of distant tissues (extravasation), the formation of small nodules of cancer cells (micrometastases), and finally the growth of micrometastatic lesions into macroscopic tumors, this last step being termed colonization. The epithelial-mesenchymal transition (EMT), a program normally occurring during embryonic development and wound healing, has become prominently implicated in this cascade. Several of the transcription factors responsible for EMT (e.g. Snail, and Slug) can directly repress E-cadherin gene expression, and have been shown in experimental models of carcinoma formation to be causally important for programming invasion; ectopic over-expression of some of these factors has even been found to elicit metastasis (Micalizzi, et al., 2010; Schmalhofer, et al., 2009). It remains to be determined whether EMT also contributes to invasion of non-epithelial tumor types,

although expression of EMT-inducing transcription factors has been observed in some cases.

Two additional, distinct modes of cancer cell invasion have been identified (Friedl and Wolf, 2008). In one, termed "collective invasion", nodules of cancer cells advance *en masse* into adjacent tissues. This is characteristic of e.g. squamous cell carcinomas; coincidentally, these cancers are rarely metastatic, suggesting that collective invasion lacks certain functional attributes to facilitate metastasis. The second mode of invasion, in which individual cancer cells gain morphological plasticity, enabling them to slither through existing interstices in the extracellular matrix, is termed "amoeboid" (Madsen and Sahai, 2010). It is not yet clear whether either of these modes of invasion employs any components of the EMT program, or whether there are still other cell-biologic pathways contributing to invasion and metastasis.

The physical dissemination of cancer cells from the primary tumor to distant tissues is only one aspect of metastasis; the other major phase of metastasis relates to the adaptation of these cells to foreign tissue micro-environments, resulting in successful colonization. Little is known about the precise steps involved in colonization. Carcinoma cells that have undergone EMT during initial invasion and metastasis, might - when no longer under the influence of EMT-inducing signals from the original tumor micro-environment, - undergo a reversal process (termed the mesenchymal-epithelial transition, or MET), resulting in the formation of new tumor colonies. The explosive metastatic growth observed in the clinic for certain cancers, soon after resection of the primary tumor, suggests that the primary tumor might release factors that initially render micrometastases dormant. On the other hand, metastases that erupt decades after treatment of the primary tumor reflect the heterogeneity of the primary tumor (see below): the disseminated cells might lack certain hallmark capabilities, such as sustained proliferative signaling in the absence of

growth factors in the new micro-environment, insensitivity to growth signals present in this new micro-environment, or induction of angiogenesis. Nutrient starvation might induce intense autophagy (see 2.5.1), causing cells to adopt a state of dormancy, which is reversed upon favorable changes in the new micro-environment.

Alternatively, metastatic dissemination may also lead to "re-seeding" of cancer cells at the site of the primary lesion. It is likely that the micro-environment at the primary tumor site is intrinsically hospitable to malignant cells that 'return home', resulting in successful recolonization. Finally, while metastatic dissemination is generally regarded as the final step in neoplastic progression, there are reports indicating that cells can disseminate remarkably early, dispersing from noninvasive premalignant lesions in both mice and humans (Coghlin and Murray, 2010; Klein, 2009). The clinical significance of this phenomenon is however yet to be established, as the ability of such premalignant cells to successfully colonize distant sites remains unproven.

Alteration of cellular metabolism

As briefly alluded to before, the onset of proliferation introduces important problems in not only the cell cycle, but in cellular metabolism as well, for each passage through the cycle requires a doubling of total biomass. Consequently, if cells are to proliferate rapidly and uncontrollably, as is the case in cancer, a profound metabolic reprogramming is required (DeBerardinis, et al., 2008).

At rest, basal levels of growth-factor signaling allow cells to take up sufficient nutrients to provide for the low levels of ATP production and macromolecular synthesis needed to maintain cellular homeostasis. In the absence of any extrinsic signals, mammalian cells lose surface expression of nutrient transporters. To survive in the absence of the ability to take up extracellular nutrients, growth-factor-deprived cells

engage in autophagic degradation of macromolecules and organelles. This is afinite survival strategy, which can ultimately result in cell death. In contrast, mitogenic signaling instructs cells to begin taking up nutrients at a high rate and to allocate them into metabolic pathways that support production of ATP and macromolecules including proteins, lipids, and nucleic acids. The resulting increase in aerobic glycolysis, de novo lipid biosynthesis, and glutamine-dependent anaplerosis, culminating in a net increase in cellular biomass (growth) and, ultimately, the formation of daughter cells, is now regarded as the seventh hallmark of tumorigenicity (Hanahan and Weinberg, 2011; Weinberg and Chandel, 2009).

These features were first observed by Otto Warburg over 80 years ago, who noted that rapidly proliferating tumor cells consume glucose at a higher rate than normal cells, secreting most of the glucose-derived carbon as lactate rather than oxidizing it completely (a phenomenon known as the 'Warburg effect') (Warburg, 1925, 1956). Many reports have since corroborated that an increase in (aerobic) glycolysis is indeed a hallmark of tumorigenicity (Gatenby and Gillies, 2004), though aerobic glycolysis itself is not unique to tumor cells, as it also occurs in rapidly proliferating primary cells. The high glycolitic rate provides several advantages for proliferating cells. It allows cells to use the most abundant extracellular nutrient, glucose, to produce abundant ATP. Notably, the glucose transporter GLUT1 is up-regulated in many human tumors (DeBerardinis, et al., 2008). Although the yield of ATP per glucose consumed is lower compared to oxidative phosphorylation, the rate of ATP production during glycolysis is higher (Pfeiffer, et al., 2001). Also, further compensating for the lower efficiency of aerobic glycolysis compared to oxidative phosphorylation, is the fact that glucose degradation provides cells with intermediates needed for biosynthetic pathways (van der Heiden, et al., 2009). There is even advantage in the clinic, where positron emission tomography (PET) exploits the increased uptake and

utilization of glucose in cancer cells by using a radio-labeled analog of glucose (^{18}F-fluorodeoxyglucose, FDG) to visualize metastatic lesions.

The molecular mechanism behind the metabolic switch observed in tumor cells is regulated by the PI3K/AKT/mTOR pathway. PI3K activation can increase glucose uptake and utilization through AKT (Elstrom, et al., 2004; Rathmell, et al., 2003); mTOR stimulation activates the transcription factor HIF-1 (Majumder, et al., 2004), which enhances glycolysis by increasing the expression of genes that encode glycolytic enzymes and glucose transporters (Semenza, 2000, 2007). Oncogenes such as Ras and Myc also stimulate glycolysis through induction of glycolytic enzymes and glucose transporters (Dang and Semenza, 1999), and activating mutations have been reported for the isocitrate dehydrogenase 1/2 (IDH) enzymes in certain types of cancer (Yen, et al., 2010). Furthermore, the PI3K/AKT/mTOR pathway also stimulates ribosome biogenesis, which is fundamental to achieve rapid cell growth and proliferation (Dufner and Thomas, 1999; Gingras, et al., 2004).

Evasion of immune destruction

Yet another particular feature of cancer cells concerns their relationship to the immune system. Ordinarily, cells of the innate and adaptive immune response cooperate to protect the body against harmful agents, including bacteria, viruses and parasites. Evidence suggests, however, that these cells also function in "tumor surveillance", in which cells and tissues are constantly monitored for nascent tumors, recognizing and eliminating incipient cancer cells. While this is obviously plausible for virus-induced cancers, it seems less so for the >80% of tumors of non-viral etiology. Still, human tumors frequently have defects in MHC class I antigen presentation (Seliger, 2008), and deficiencies in the development or function of cytotoxic T lymphocytes (CTLs), helper T cells or natural killer (NK) cells each led to demonstrable increases in cancer incidence in mouse

models (Kim, et al., 2007; Teng, et al., 2008). Clinical epidemiology also increasingly supports the existence of anti-tumoral immune responses in human cancer; for example, patients with colon and ovarian tumors that are heavily infiltrated with CTLs and NK cells have a better prognosis than those lacking this abundant immune response (Bindea, et al., 2010). Furthermore, cancer cells may paralyze infiltrating CTLs and NK cells by secreting e.g. TGFβ (Yang, et al., 2010), or suppress their actions by recruiting inflammatory cells that are actively immunosuppressive, such as regulatory T cells and myeloid-derived suppressor cells (MDSC) (Mougiakakos, et al., 2010; Ostrand-Rosenberg and Sinha, 2009).

Another class of cells pertaining to the immune system comprises the dendritic cells (DCs). As antigen-presenting cells, DCs play a central role in both innate and adaptive immunity. DCs can be found in tumors in both humans and mice; however, cancer cells have been shown to suppress DCs through the expression of cytokines such as IL-6 and -10, and VEGF (which, coincidentally, also stimulates angiogenesis). Alternatively, tumors may condition DCs to form suppressive T cells, and studies have shown that in multiple myeloma, DCs even support clonogenic growth (Steinman and Banchereau, 2007, and references therein). Thus, much like certain infectious agents (e.g. HIV), cancer cells have developed strategies to evade, and in some instances even exploit, DCs.

Taken altogether, the data imply that anti-tumor immunity might be a significant barrier to tumor formation and progression, imposing upon tumor cells the need to acquire the ability to either evade immune suppression, or adapt it to promote proliferation.

Genomic instability

Acquisition of the features discussed above depends in large part on a succession of alterations in the genomes of neoplastic cells. This

entails mutations, but also epigenetic modifications. Ordinarily, genome maintenance systems (often referred to as the caretakers of the genome) ensure that the rates of spontaneous mutations per cell cycle are very low. Additionally, as discussed above, p53, the "guardian of the genome", plays a central role in the surveillance systems that normally monitor genomic integrity and inhibit proliferation of genetically damaged cells. Analysis of cancer cell genomes has shown that many tumor cells appear to specifically target the caretakers and guardians of the genome for deletions and inactivating mutations, further accelerating the accumulation of tumor-promoting genomic alterations. Conversely, other genomic regions, harboring genes whose expression favors neoplastic progression, are often amplified in cancer cells. Genomic imbalance is thus an enabling characteristic, exploited by cancer cells to acquire the hallmark capabilities required for malignant transformation.

Telomerase has ambiguous roles in this regard: in the absence of telomerase expression, sustained proliferation results in loss of telomeric DNA, leading to end-to-end fusions and general karyotypic instability. While the resulting genetic alterations could be advantageous to the cancer cell, they may also induce cellular senescence. Increased expression of telomerase, while bypassing senescence, may reduce genomic instability and delay neoplastic progression; prolonged expression of telomerase may again lead to genomic imbalance due to fusion and breakage of excessively elongated telomeres.

The immune system and other cells of the tumor micro-environment

As discussed before, some tumors are densely infiltrated by cells of both the innate and adaptive arms of the immune system. What's more, it's becoming increasingly clear that practically every neoplastic lesion contains immune cells – ranging from subtle infiltrations to gross inflammations. This is largely though to reflect an attempt by

the immune system to eradicate cancerous cells. However, the tumor-associated inflammatory response has been shown to have a paradoxical effect, enhancing tumorigenesis and progression, in fact helping incipient neoplasias to acquire hallmark capabilities.

Inflammatory cells supply growth factors to sustain proliferative signaling, survival factors limiting cell death, pro-angiogenic factors, extracellular matrix-modifying enzymes facilitating angiogenesis, invasion and metastasis, and EMT-inducing signals (DeNardo, 2010; Grivennikov, 2010; Karnoub and Weinberg, 2006, 2007; Kessenbrock, et al., 2010; Qian and Pollard 2010), and have even been shown to release mutagenic factors, promoting genomic imbalance (Grivennikov, 2010). Concurrently, inflammation is in some cases evident at the earliest stages of neoplastic progression, and is demonstrably capable of fostering the development of incipient neoplasias into full-blown cancers (Qian and Pollard, 2010: de Visser, 2006). The tumor-stroma interaction is not one-sided: not only do cancer cells secrete factors to suppress elimination by the cells of the immune system, but they have also been shown to stimulate these cells. In an experimental model of metastatic breast cancer, the cancer cells secreted CSF-1, stimulating tumor-associated macrophages, while the latter reciprocated by supplying epidermal growth factor (EGF) to the breast cancer cells (Qian and Pollard, 2010).

Evidently, these interactions also extend to the other cells in the tumor micro-environment. For contrary to earlier views, tumors are now regarded as complex, organized networks of heterogeneous, specialized cells – comparable to organs. Besides the cells of the immune system, these include endothelial cells and pericytes, which form the tumor-associated vasculature, as well as fibroblasts and other stromal cells.

Another important constituent of the tumor micro-environment concerns the so-called "cancer stem cells" (CSCs). Traditionally, tumors have been portrayed as reasonably homogeneous cell populations – principally arising from a single cell that managed to acquire the hallmark capabilities - until relatively late in the course of tumor progression, when hyperproliferation combined with increased genetic instability would spawn distinct clonal subpopulations. However, there is increasing evidence that certain cancer cells assume a stem cell-like character. CSCs, like their normal counterparts, may self-renew as well as spawn more differentiated derivatives. The origins of these CSCs is not entirely clear, though it is proposed that they arise either through de-differentiation, or through oncogenic transformation of normal tissue stem cells (Cho and Clarke, 2008; Lobo, et al., 2007). Additionally, CSCs have been shown to express markers of their corresponding normal tissue stem cells (Al-Hajj, et al., 2003). They were originally implicated in the pathogenesis of hematopoietic malignancies, but have now also been identified in e.g. breast carcinomas and neuroectodermal tumors. In fact, induction of the EMT program in certain model systems has been shown to induce many of the defining features of stem cells (Mani, et al., 2008).

One important implication of the above-discussed, recently acquired knowledge on the tumor micro-environment, is that all the core hallmark capabilities might not need to reside within a single cell. For instance, the ability to negotiate the invasion-metastasis cascade may be acquired in certain cancers via inflammatory cells in their micro-environment, without the requirement that the cancer cells themselves undergo additional mutations beyond those that were needed for primary tumor formation. Another is that the dynamic interactions between cancer cells and their micro-environment, and the development of CSCs, complicates not only the elucidation of the mechanisms of cancer pathogenesis, but also the development of novel therapies to successfully target primary and metastatic tumors.

2.4 Oncogenic transformation: the making of a human tumor cell

Regardless of the many remaining uncertainties, the set of cancer-typical traits discussed above does allow for a tentative model of oncogenic transformation (Figure 2.3). Experiments using the viral oncoproteins Simian Virus 40 (SV40) large and small T antigens have elegantly demonstrated that full malignant transformation of human cells can be achieved in a limited number of steps, requiring (Hahn and Weinberg, 2002b):

- Oncogenic activation of Ras, e.g. through activating mutations, conferring growth signal autonomy;
- Bypassing replicative senescence and evasion of apoptosis by the introduction of SV40 LT, which binds to and inhibits the functions of pRb and p53, respectively (Ali and DeCaprio, 2001);
- Activation of telomerase to achieve immortalization;
- Co-expression of SV40 ST, which associates with PP2A and alters its cellular function (Yu, et al., 2001). Though PP2A has many cellular functions and has been shown to be an important tumor suppressor, exactly how inhibition of PP2A contributes to malignant transformation remains unclear (Mumby, 2007).

Intriguingly, while the fifth and sixth hallmarks are not required for malignant transformation, but rather promote continued proliferation, invasion and metastasis once the tumor has been formed, the seventh hallmark is indeed activated by Ras. Similarly, the eighth proposed hallmark appears not to be required for initial malignant transformation, though one might speculate that the SV40 antigens could perhaps either trigger the activation of the immune system, eliciting tumor-promoting inflammation, or actively suppress antigen presentation, aiding in immune escape of infected cells. Furthermore, owing to the inhibition of pRb and p53, cells are predisposed to genomic instability, facilitating the acquisition of the remaining hallmarks and thus further neoplastic progression.

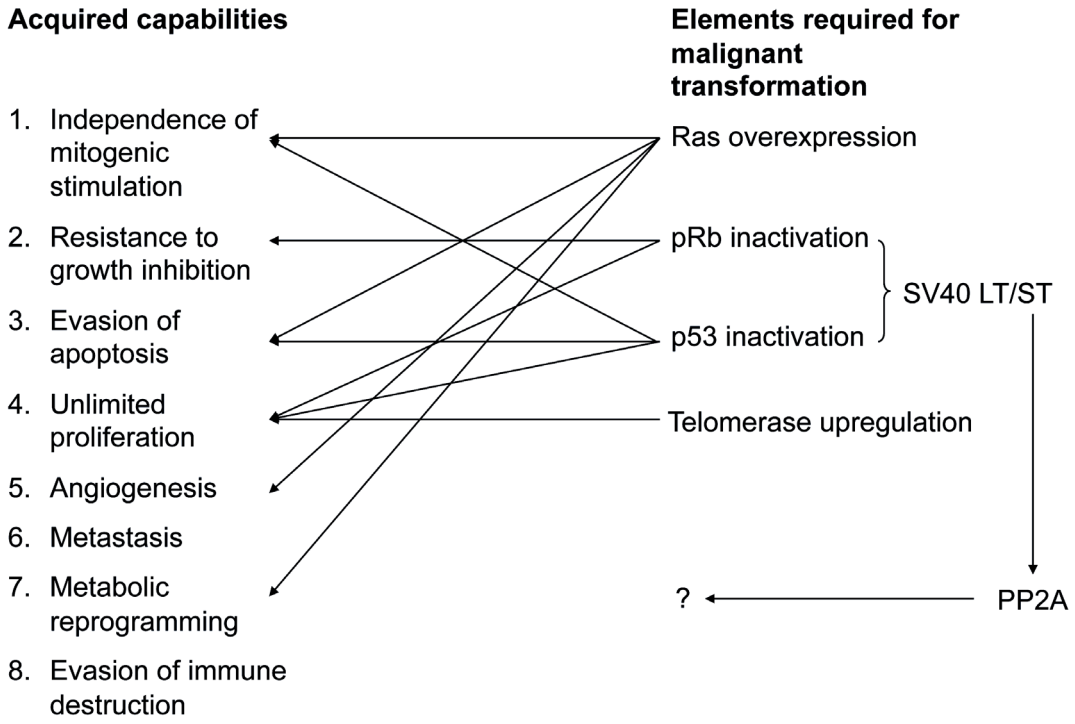

Figure 2.3 Experimental findings demonstrate that only a few steps are necessary for malignant transformation of human cells. Over-expression of Ras confers independence from mitogenic signaling, while inactivation of the tumor suppressors pRb and p53 confer immortalization, which is sustained by upregulation of telomerase. Ras over-expression also induces angiogenesis and the seventh proposed hallmark, namely the metabolic switch, which is postulated to be required to provide the energy and nutrients necessary for rapid cellular proliferation. PP2A inactivation by SV40 ST has been demonstrated to be required for full malignant transformation, though how this contributes to tumorigenesis has yet to be elucidated. Adapted from Hahn and Weinberg, 2002b.

2.5 Killing tumor cells in the 21st century

Cancer is traditionally treated by debulking through surgery, and killing any remaining cells by a combination of radio- and chemotherapy. As the conventional therapies have been designed to target rapidly proliferating cells in general, and do not target the tumor cells specifically, they are also toxic to normally rapidly proliferating cells, causing serious side-effects, such as anemia, and suppression of the immune system. Furthermore, they rely heavily on the induction of apoptosis, whereas, as discussed previously, cancer cells typically accumulate alterations to the apoptotic machinery, conferring on them the ability to evade apoptosis. Recent

understanding of the molecular pathogenesis of cancer has led to the development of targeted therapies, and increasing attention is being directed towards other types of cell death, including autophagy, mitotic catastrophe, necrosis and senescence. The various pathways leading to cell death are discussed in section 2.5.1, and the novel anticancer strategies designed to effectuate cancer cell death are presented in section 2.5.2.

2.5.1 Cell death pathways and response to antitumor therapy

The various modes of cell death have long been classified according to their morphological features (Kroemer, et al., 2009). Recent breakthroughs in cell death research have, however, allowed for the tentative introduction of a novel characterization based on measurable biochemical features (Galluzzi, et al., 2011). Both the morphological and biochemical features of the various cell death types are summed up in Table 2-2 and schematically depicted in Figure 2.4. Even though the various modes of cell death are discussed as separate entities, one must keep in mind that many interconnections exist: e.g., the apoptosis and autophagy pathways share a number of components (Maiuri, et al., 2007), while autophagy is required to mediate the senescence transition (Young, et al., 2009).

Apoptosis

Apoptosis is the term for programmed cell death, in which the cell membrane is disrupted, the cytoplasmic and nuclear skeletons are broken down, the nucleus is fragmented, chromosomes are degraded, and the shriveled cell corpse, neatly packaged, is engulfed by nearby cells and disappears, without eliciting an inflammatory response (Kroemer, et al., 2009).

The apoptotic machinery, depicted in Figure 2.4A, consists of sensor proteins and a family of effector proteins called caspases (Kurokawa

Table 2-2. The morphological features of the different modes of cell death. Adapted from Wlodkowic, et al., 2010 and Galluzzi, et al., 2011. MAP1LC3, micro-tubule-associated protein 1 light chain 3; SQSTM1, sequestosome 1

Type of cell death	**Morphological features**	**Distinctive biochemical features**
Apoptosis	Rounding-up of the cell Reduction of cellular and nuclear volume Nuclear fragmentation Plasma membrane blebbing Minor modification of cytoplasmic organelles Engulfment by resident phagocytes *in vivo*	Internucleosomal DNA fragmentation Phosphatidylserine exposure Intrinsic apoptosis - caspase-dependent, cytochrome c release - caspase-independent Extrinsic apoptosis - death receptor signaling, caspase-8/-10 activation - dependence receptor signaling, caspase-9 activation
Autophagy	Lack of DNA fragmentation Accumulation of (double-membraned) autophagic vacuoles Little or no uptake by phagocytic cells *in vivo*	Increased lysosomal activity Initially perceived as caspase-independent although recent reports indicate cross-talk with apoptosis MAP1LC3 lipidation SQSTM1 degradation
Necrosis	Dissolution of chromatin Swelling of cytoplasm and cytoplasmic organelles Rupture of plasma membrane	Lack of caspase cascade activation RIP1/3 activation
Mitotic catastrophe	During mitosis: multiple micronuclei, aberrant mitotic spindles Following mitotic failure: formation of giant polykaryons	Mitotic arrest Caspase-2 activation (in some cases) p53/p73 activation (in some cases)
Senescence	Appearance of characteristic heterochromatic foci Flattened cytoplasm Increased cellular granularity	Initiated by telomere shortening Activation of SA-β-gal Caspase-independent

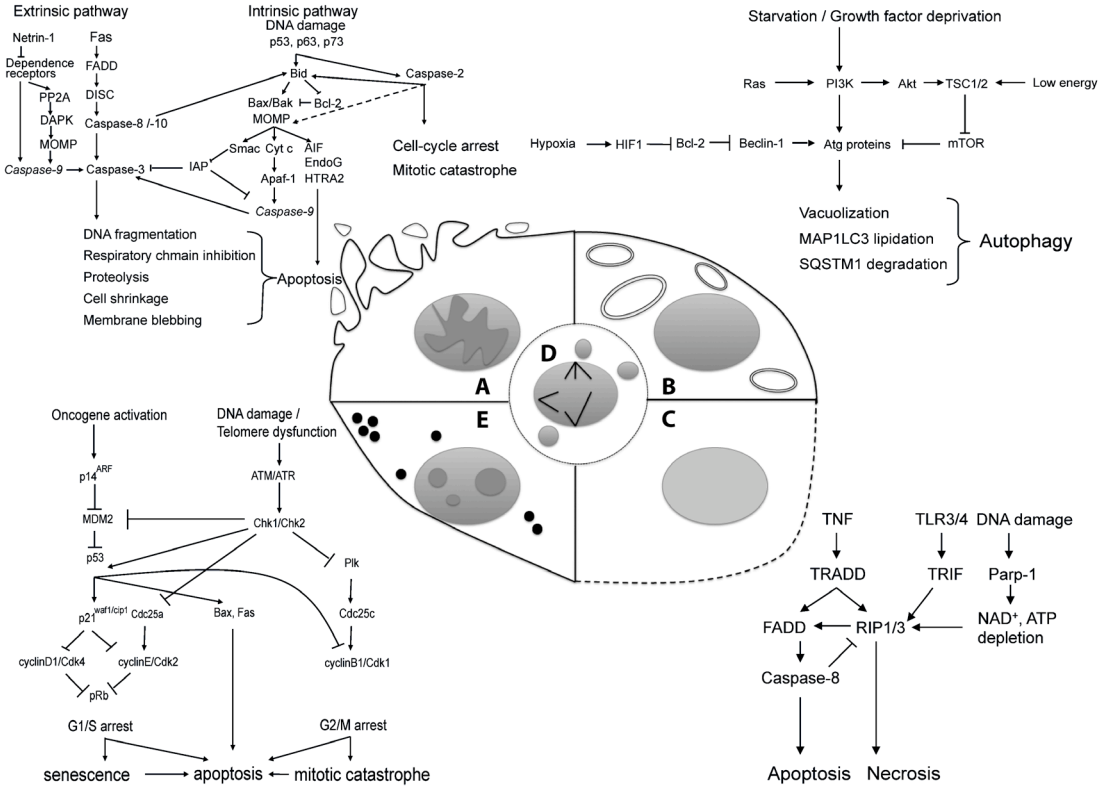

Figure 2.4. Schematic depiction of the various modes of cell death, and a general overview of the most important molecular players involved, also indicating the cross-talks existing between the different pathways. **A.** Apoptosis is characterized by nuclear chromatin condensation and fragmentation, cell shrinkage and blebbing of the cytoplasmic membrane. It can be induced extrinsically by stimulation of death receptors, e.g. FADD and insufficient survival signaling, or intrinsically, by e.g. DNA damage. Both pathways converge on the activation of the executioner caspase, caspase-3; however, DNA damage-induced activation of caspase-2 can also result in cell cycle arrest and mitotic catastrophe. Release of AIF, EndoG, and HTRA2 proteins from the mitochondria can also induce caspase-independent apoptosis. MOMP, mitochondrial outer membrane permeabilization. **B.** Autophagic cell death is characterized by the appearance of double-membraned autophagic vacuoles and the lack of chromatin condensation. Autophagy is induced by starvation and/or growth factor deprivation, which stimulates PI3K to induce the formation of autophagosomes comprising Beclin-1 and various Atg proteins. Other cellular stress signals, such as hypoxia and low energy also stimulate autophagy, respectively by removing Bcl-2 sequestration of Beclin-1, and mTOR suppression of autophagosome assembly. **C.** Necrotic cell death is characterized by chromatin dissolution, cytoplasmic swelling and rupture of the cell membrane. The kinase RIP1 and its homolog RIP3 are central players in this process, and induce necrosis in the case of caspase inhibition. **D.** Mitotic catastrophe is the result of damaged DNA and aberrant mitotic spindle formation. Abrupt interruption of mitosis at metaphase/anaphase results in the formation of multiple micronuclei. Otherwise, in the case of mitotic failure, the spindle is disassembled and cells enter G1 without having undergone cytokinesis, forming giant polykaryons (not depicted). **E.** DNA-damage- and oncogene-induced senescence is characterized by the appearance of characteristic heterochromatic foci, cytoplasmic granules, and flattening of the cytoplasmic membrane. See text for further details.

and Kornbluth, 2009). Apoptosis can be initiated by two distinct pathways, respectively conveying intra- and extracellular stress signals. Intracellular stress signals, such as growth factor withdrawal, DNA damage, oxidative stress or oncogene activation, lead to release of cytochrome c from the intermembrane space of the mitochondria to the cytoplasm. This process is tightly regulated by the Bcl-2 family of both pro- and anti-apoptotic proteins, and results in the activation of caspase-9. The extrinsic pathway is activated in one of two ways: either by the binding of death-inducing ligands, such as Fas and TNFα, inducing formation of the death-inducing signaling complex (DISC), and activation of caspase-8 and -10, or alternatively, through the actions of "dependence receptors", when the concentration of their specific ligands fall below a certain threshold (Mehlen and Bredesen, 2011). Both apoptotic pathways lead to activation of the executioner caspases, caspase-3, -6 and -7, which are the main proteases responsible for cellular degradation.

In addition, experiments with caspase inhibitors, wherein cell death could be delayed but not inhibited, led to the proposal of a caspase-independent mode of intrinsic apoptosis. This would entail the release of AIF, EndoG and HTRA2 from the mitochondria in response to intrinsic stress signals, leading to large-scale DNA fragmentation and cleaving of a wide array of proteins, including cytoskeletal proteins.

The last stage of apoptosis involves the uptake of apoptotic cells by phagocytosis. This process is initiated by externalization of phosphatidylserine on the surface of apoptotic cells, facilitating recognition, uptake and removal of apoptotic cell debris by phagocytes.

Autophagy

Autophagy is characterized by the sequestration of cytoplasmic material (proteins and organelles) within autophagosomes for bulk

degradation by lysosomes (Kroemer, et al., 2009). Typically, autophagic cell death occurs in the absence of chromatin condensation, but is accompanied by massive autophagic vacuolization of the cytoplasm. These so-called "autophagosomes" originate from two conjugation systems, involving the autophagy-associated Atg proteins (de Bruin and Medema, 2008) (Figure 2.4B). In fact, lipidation of Atg8 (MAP1LC3) is a defining biochemical feature of autophagy, as is degradation of the autophagic substrate sequestosome 1 (SQSTM1) (Table 2-2). The autophagic pathway is regulated by the PI3K/AKT/mTOR pathway (Petiot, et al., 2000; Wang and Klionsky, 2003), which, coincidentally, is also responsible for the metabolic switch observed in rapidly proliferating cells (the seventh hallmark of cancer).

Rather than being simply a cell death pathway, autophagy is actually quite important for cell survival, providing an alternative source of nutrients (Klionsky and Emr, 2000). In yeast, autophagy is induced under nutrient-limiting conditions as a mechanism to survive; however, in *Drosophila melanogaster*, autophagic structures are formed during morphogenesis, corroborating its role in cell death (Baehrecke, 2003). It has therefore been considered that, under conditions of cellular stress, autophagy might start as an adaptive response in order to enhance cell survival, but that, beyond a certain threshold, it can result in cell death. Importantly, some reports indicate that cells displaying features of autophagic cell death can still recover upon withdrawal of the death-inducing stimulus (Boya, et al., 2005).

During cellular transformation, autophagy may prevent a normal cell from becoming a malignant one by degrading damaged organelles and thereby reducing cellular stress, or by degrading specific proteins that enhance tumor formation (Jin and White, 2007; Mathew, et al., 2007). It may also limit chromosome instability and thereby tumor

progression (Mathew, et al., 2007). Alternatively, autophagy may prevent tumorigenesis by killing premalignant cells (Karantza-Wadsworth, et al., 2007). Besides its potential tumor-suppressive roles in the early stages of tumorigenesis, autophagy has also been proposed to play a tumor-promoting role during the later stages of tumor growth (Amaravadi, et al., 2007; Lum, et al., 2005). In this case, autophagy protects cells against stressful conditions. Notably radio- and chemotherapy treatment can induce autophagy, leading to a state of reversible dormancy, enabling the resistance, persistence and regrowth of tumors (Apel, et al., 2009; White and DiPaola, 2009).

Necrosis

Necrotic cell death is characterized by cellular swelling, rupture of the plasma membrane and subsequent loss of intracellular contents, often provoking an inflammatory response (Kroemer, et al., 2009). As opposed to apoptosis, necrosis has long been considered to be an uncontrolled form of cell death. However, evidence is accumulating that the execution of necrotic cell death may be finely regulated by death domain receptors and Toll-like receptors, and is dependent on the activity of the kinase RIP1 and its homolog RIP3 (Festjens, et al., 2007) (Figure 2.4C).

Neither the precise role of the kinase activity of RIP1 nor its downstream targets are known. Previously, it was shown that mitochondria-produced reactive oxygen species (ROS) are important players in the execution of necrotic cell death (Festjens, et al., 2006). Therefore, it is conceivable that RIP1 directly or indirectly targets mitochondria. Indeed, in tumor necrosis factor (TNF)-stimulated cells, RIP1 translocates to the mitochondria. In addition, RIP1 has also been shown to be essential for TNF-induced production of ceramide, the latter mediating TNF-induced caspase-independent cell death. As the phospholipase cPLA2 contributes to TNF-induced necrosis (Thon, et al., 2005), it is conceivable that a RIP1-cPLA2-acid sphingomyelinase

pathway may lead to necrotic cell death. Because inhibition of ceramide accumulation clearly diminished caspase-independent cell death but not as completely as inhibition of RIP1, ceramide obviously may represent a central factor, but most likely not the only one, transmitting the death signals generated by RIP1 in response to TNF.

Notably in some studies, RIP1-dependent autophagic cell death instead of necrosis was observed (Yu, et al., 2006). However, the induction of autophagic cell death was much slower than the induction of death receptor-induced necrotic cell death. Thus, whether necrosis or autophagy ensues when apoptosis is inhibited, will surely depend on cells and circumstances.

Necrosis can also be induced through DNA damage (Festjens, et al., 2006). This type of cell death is mediated by PARP-1, a protein involved in DNA damage repair. Activation of PARP-1 catalyzes the hydrolysis of NAD+ into nicotinamide and poly-ADP ribose, causing depletion of NAD+. This results in cellular energy failure and caspase-independent death of different cell types.

Unfortunately, the inflammatory response which accompanies necrotic cell death (in contrast to apoptosis and autophagy), can in fact promote neoplastic progression, given that the inflammatory cells can foster proliferation, angiogenesis and tissue invasion and metastasis (see paragraph 2.3). Additionally, necrotic cells can release factors like IL-1α, which can directly stimulate viable neighboring cells to proliferate, again facilitating neoplastic progression.

Mitotic catastrophe

Mitotic catastrophe is a type of cell death that follows aberrant mitosis, occurring either during or shortly thereafter. In mammalian cells, and particularly in tumor cells, mitotic catastrophe is mainly associated with activation of the G2/M cell cycle checkpoints for DNA

damage/structure and spindle assembly, and involves numerous players involved in these checkpoints, including Chk2, cyclinB/Cdk1, and members of the p53 family, including p53 and the p73 variant TAp73 (Figure 2.4D) (de Bruin and Medema, 2008). Following mitotic catastrophe, cells are ultimately killed by engaging the apoptotic or necrotic pathways, or by induction of cellular senescence (Figure 2.4E) (Galluzzi, et al., 2011).

At least two subtypes of mitotic catastrophe can be distinguished (Castedo, et al., 2004). First, mitotic catastrophe can kill the cell during or close to metaphase, in a p53-independent manner involving the activation of caspase-2. Second, mitotic catastrophe can occur after failed mitosis, in a partially p53-dependent manner involving the activation of the polyploidy checkpoint in G1. Even though mitotic catastrophe is accompanied by chromatin condensation and mitochondrial release of apoptosis-inducing factor and cytochrome c, which are key features of apoptosis, there are a number of fundamental differences. Importantly, it has been shown that over-expression of Bcl-2 does not block and might actually enhance mitotic catastrophe (Lock and Stribinskiene, 1996). Cell death occurring during the metaphase-to-anaphase transition is characterized by the activation of caspase-2, which is activated in the nucleus in response to DNA damage (Lassus, et al., 2002; Paroni, et al., 2002), and is a process that cannot be inhibited by Bcl-2 (Peart, et al., 2003; Read, et al., 2002; Robertson, et al., 2002).

Senescence

Analogously to the replicative senescence induced in primary cells as a result of shortened telomeres, treatment of malignancies may result in a permanently growth-arrested state (Gewirtz, et al., 2008). This is termed 'accelerated senescence', and is sensed as a permanent state of DNA damage, while the cell remains viable and metabolically active (Figure 2.4E). DNA damage signaling activates the p53 and pRb

proteins (or their respective family members, as p53 and pRb function are often lost during tumorigenesis), which respectively results in first a temporary, then a prolonged arrest in G1. Senescent cells characteristically display senescent associated DNA damage foci (SDF) and senescent associated heterochromatin foci (SAHF) (Campisi and d'Adda di Fagagna, 2007); SAHF are often found at the promoters of E2F target genes where they are thought to inhibit transcription, thereby enforcing growth arrest (Narita, et al., 2003).

2.5.2 Novel approaches to the treatment of cancer

The success of anticancer therapies depends on their ability to distinguish between normal and cancer cells and specifically exert their toxic effect on the malignant cells. Novel anticancer strategies therefore involve a targeted approach, utilizing knowledge of the cancer hallmarks and enabling characteristics discussed above, and the tumor suppressor and oncogenic pathways involved. Accordingly, the following strategies will be discussed:

- inhibition of growth signaling pathways
- induction of programmed cell death
- disruption of telomere maintenance and, hence, cellular immortalization
- targeting the tumor and its micro-environment to prevent angiogenesis and metastasis
- attenuation of tumor cell metabolism
- targeting cancer cells for immune destruction
- exploiting genomic instability
- proteins selectively killing tumor cells

Clinical experience with therapies selectively targeting only one each of these characteristics has shown that the effect is often transitory. This suggests the existence of at least some (partial) redundancy, in the form of multiple pathways governing each capability, and/or an adaptive shift from one capability to another, facilitated by genomic

instability and the tumor micro-environment. Hence, successful cancer therapies must comprise a combination of modalities.

Targeting growth signaling pathways in cancer

The first two acquired capabilities discussed in section 2.3 concerned self-sufficiency in growth signaling and insensitivity to growth-inhibitory signaling. Thus, growth-signaling pathways, and especially the receptor proteins, are interesting targets for anticancer strategies (Christoffersen, et al., 2009). Therapeutic approaches involve both hormone therapy and monoclonal antibodies. For example, targeting the EGF dependent signaling pathway has been successfully applied in the clinic. One of the receptors in this pathway is Her2 (Her2/neu, ErbB2), and it has been shown to be overexpressed in 20-25% of breast tumors. Targeting this receptor via the antibody Herceptin (trastuzumab) has proven to be very effective in the treatment of this type of cancer (Chang, 2010).

Another approach involves the development of Cdk inhibitors to halt the cell cycle, with several compounds already being evaluated in clinical trials (De Falco and De Luca, 2010).

Inducing programmed cell death

Overturning the cancer cell's apoptosis blockade has been an appealing approach in the design of anti-cancer therapies. For example, in cells where p53 function has been lost, this might be substituted for by activation of the p53 family member p73, either alone or in combination with other anti-cancer therapies (El-Rifai and Zaika, 2008; Vilgelm, 2008). The same might also be achieved by inhibition of the negative apoptosis regulator Bcl-2 (Kang and Reynolds, 2009), e.g. through so-called BH3-mimicking compounds (Chonghaile and Letai, 2008). Another promising approach is the development of small therapeutic compounds, referred to as Smac mimetics, which are designed to block the function of members of the

inhibitor of apoptosis (IAP) protein family (Gyrd-Hansen and Meier, 2010). Alterations in IAPs are found in many types of human cancer and are associated with chemoresistance, disease progression and poor prognosis (Hunter, et al., 2007; LaCasse, et al., 2008). Consistent with the idea that different types of cancer cells are dependent on ("addicted to") IAPs for their survival, the inactivation of IAPs, particularly when combined with other treatments, results in the death of most tumour cells *in vitro*. Though inactivation of IAPs does not seem to be detrimental to normal cells, loss of IAPs is also associated with the development of certain types of cancer. Several compounds are therefore currently being assessed for safety in phase I clinical trials.

As induction of the other modes of cell death may cause undesirable tumor-promoting effects, studies to define optimal strategies to modulate and exploit these other forms of cell death for cancer therapy are still ongoing. For instance, several autophagy modulators are currently being investigated (Chen and Karantza, 2011), and mechanisms being considered for the induction of senescence include restoration and/or promotion of p53 function, modulation of the cell cycle through Cdk inhibitors, and inhibition of telomerase action (see below) (Nardella, et al., 2011).

Attacking cellular immortalization via disruption of telomere maintenance

While normal human somatic cells do not or only transiently express telomerase and therefore shorten their telomeres with each cell division, most human cancer cells typically express high levels of telomerase and show unlimited cell proliferation (acquired capability 4). Telomerase is thus an attractive therapeutic cancer target, and novel anti-cancer strategies include the direct targeting of components of telomerase: the protein component hTERT or RNA component hTR (Phatak and Burger, 2007). Examples of such agents include the small

molecule hTERT inhibitor BIBR1532 and Imelstat (GRN163L), a thio-phosphoramidate oligonucleotide targeting the template region of hTR as a "template antagonist" (Kelland, 2007). Anti-tumor effects of both compounds have been observed in cell lines and, particularly for Imelstat, also in xenografted human tumors in mice. Imelstat treatment of human glioblastoma tumor-initiating cells *in vitro* led to progressive telomere shortening, reduced rates of proliferation, and eventually cell death (Marian, et al., 2010). In combination with radiation and the DNA alkylating agent temozolomide, Imelstat had a dramatic effect on cell survival and activated the DNA damage response pathway. *In vivo*, chronic systemic treatment produced a marked decrease in the rate of xenograft subcutaneous tumor growth.

Effects of anti-telomerase treatment are largely dependent upon initial telomere length, which can result in a substantial lag before antitumor activity is observed in tumors possessing relatively long telomeres. An alternative approach is therefore to target the telomere itself (Telomere Targeting Agents, TTAs) (Kelland, 2005). Several classes of small molecules have been described that induce the G-rich single-stranded overhang of telomeric DNA to fold into 4-stranded G-quadruplex structures. Such folding is incompatible with telomerase function and may induce rapid telomere uncapping. These molecules have shown potent telomerase inhibition in nanomolar concentrations *in vitro* and the rapid induction of senescence in cancer cells. The TTA BRACO19 has demonstrated single agent activity against human tumour xenografts with anti-tumour effects apparent from only 7 days of treatment.

So far, Imelstat is the only drug of its class in clinical trials. In the near future, it is expected that other direct telomerase targeted agents as well as those targeting telomeres (e.g., AS1410 based on BRACO19) will enter Phase I clinical trials (Parkinson and Minty, 2007).

Targeting angiogenesis and metastasis

As discussed above, tumor growth requires the malignant cell to be able to e.g. form new vasculature, as well as produce autologous growth factors. These characteristics need not be united in a single cell, as cells in the tumor micro-environment can contribute to neoplastic progression by providing the necessary factors, aiding not only in e.g. the angiogenic switch, but also in fostering tissue invasion and metastasis.

Furthermore, the cancer stem cells (CSCs) found in many tumors have important properties that must be considered in the development of effective cancer therapies. Their relative quiescence allows them to escape cell death by therapies that attack only rapidly proliferating cells. Also, their ability for self-renewal allows them to recover from the anti-cancer attack, repair their DNA and again initiate the formation of a new tumor. Thus, even if the cancer appears to be cured, the CSCs survive, and in time the tumor reappears. Furthermore, as CSCs are able to modify the tumor microenvironment, providing trophic factors to support tumor growth, they harbor a considerable potential for recurrence, as well as successful colonization of distant metastases.

Therapeutic strategies that could be employed to target angiogenesis and metastasis in general (acquired capabilities 5 and 6), and cancer stem cells in particular, are:

- anti-angiogenic therapy, e.g. through inhibition of VEGF using the monoclonal antibody bevacizumab, to reduce the vasculature, thereby depleting the tumor of oxygen and essential nutrients (Cook and Figg, 2010)
- blocking adhesion between cells and to the extracellular matrix to prevent successful colonization of metastases, e.g. by targeting tumor-specific cadherins and integrins (Blaschuk and Devemy, 2009; Desgrosellier and Cheresh, 2010)

- another way to prevent metastasis might be through blocking the EMT chemotaxis pathways that are active in the tumor and its micro-environment (Nieto, 2011; Roussos, et al., 2011)
- inducing differentiation, e.g. through BMP signaling to revert the capacity of CSCs to form tumors and increase their sensitivity to therapy (Ghotra, et al., 2009)
- targeting self-renewal and quiescence, e.g. through PTEN or Wnt signaling pathways, to decrease the CSC population (Ghotra, et al., 2009)

Targeting cancer cell metabolism

In addition to acquiring a complex array of genetic changes, tumor cells develop an alteration in the metabolism of glucose and oxygen (acquired capability 7). As this altered metabolism does not appear to be subject to the high genetic variability of tumors, it may represent a more reliable target for cancer therapy.

The altered metabolism of cancer cells is associated with increased glycocolytic activity and repression of oxidative phosphorylation. The harmful effects of the concurrent increase in H_2O_2 production is counterbalanced by the increase in glycolytic activity, creating a self-reinforced loop. This loop can be interrupted by increasing the cellular levels of H_2O_2 (using e.g. pro-oxidant agents), or by attenuating glycolysis (using glycolysis inhibitors), or a combination of both (López-Lázaro, 2010).

In fact, many anticancer agents, such as paclitaxel, doxorubicin and arsenic trioxide, produce H_2O_2 (Alexandre, et al., 2006; Jing, et al., 1999; Ubezio and Civoli, 1994). Also, using several cancer and normal cell lines, Chen, et al. (2005) observed that high concentrations of ascorbic acid selectively killed cancer cells and that this effect was mediated by H_2O_2. Several glycolysis inhibitors have shown anticancer effects (e.g. 2-deoxy-D-glucose, lonidamine, 3-bromopyruvate and

dichloroacetate) and some of them have already entered clinical trials (Chen, et al., 2007; Gatenby and Gillies, 2007; Lopez-Lazaro, 2008; Martin, 2006; Pelicano, 2006; Xu and Huang, 2006). For example, it has been shown that dichloroacetate, a known glycolysis inhibitor that has been used in humans for decades in the treatment of lactic acidosis and inherited mitochondrial diseases, induced marked anticancer effects in mice (Bonnet, et al., 2007). Other strategies that might also be used to exploit the increased glycolytic activity of cancer cells and selectively kill these cells, are inhibition of the Na+/K+-ATPase pump (e.g. by cardiac glycosides), or of cellular systems involved in the extrusion of (acid-death inducing) protons from the cytosol.

Because of the dual role of autophagy in cancer, it is difficult to predict whether inhibition or stimulation of autophagy may result in tumor cell death (Apel, et al., 2009). Preclinical studies with chloroquine, which inhibits lysosome acidification and thereby autophagy, in conjunction with alkylating agents, showed remarkable efficacy inhibiting tumour growth in mice (Amaravadi, et al., 2007). Alternatively, promoting autophagy might also be expected to limit tumor progression. Hence, before autophagy can be targeted for the treatment of cancer, further studies investigating the dichotomy of its roles in tumor prevention and promotion are warranted (Rosenfeldt and Ryan, 2009).

Another aspect of the metabolic switch that takes place during neoplastic progression concerns the concurrent increase in protein production, and hence, ribosome biogenesis. Though this has long remained a largely unexploited target in cancer therapy, increasing attention is being paid to inhibitors of rRNA synthesis for the development of novel therapeutic strategies (Drygin, et al., 2010). For example, CX-3543, a small molecule nucleolus-targeting agent that selectively disrupts nucleolin/rDNA complexes in the nucleolus,

thereby inhibiting Pol I transcription and inducing apoptosis in cancer cells, is currently being evaluated for treatment of carcinoid/neuroendocrine tumors in a phase II clinical trial (Drygin, et al., 2009). Some classic anticancer therapeutics, including cisplatin and 5-fluorouracil, have even been shown to exert their activity, at least partially, through disruption of ribosome biogenesis (Ghoshal and Jacob, 1997; Jordan and Carmo-Fonseca, 1998).

In addition, studies indicate that many common and specialized mRNA export factors, including CRM1 and eukaryotic translation initiation factor 4E (eIF4E), are disregulated in cancer, making them also attractive therapeutic targets (Siddiqui and Borden, 2011). Indeed, specific targeting of the eIF4E-dependent mRNA export pathway in a phase II proof-of-principle trial with ribavirin led to impaired eIF4E-dependent mRNA export, correlating with clinical responses including remissions in leukemia patients (Assouline, et al., 2009).

Immune destruction of cancer cells

The ability of cells of the immune response system to infiltrate tumors presents a unique opportunity for combating cancer cells. Tumors are replete with potential antigens, which can become immunogenic when presented by DCs, activating the different arms of cell-mediated resistance (Steinman and Banchereau, 2007). This means that the resulting immune attack can encompass multiple targets, diminishing the cancer's chances of immune escape. Following recognition of tumor-specific antigens, T lymphocytes exert their cytotoxic effects on tumor cells via the extrinsic apoptosis pathway, involving Fas, and via the secretion of perforin and serine proteases granzyme A and B (Pardo, et al., 2004). Perforin is a transmembrane pore-forming molecule, which allows granzyme A and B to enter the target cell and induce apoptosis. Granzyme A activates caspase-independent pathways by inducing single-stranded DNA damage, while granzyme B

directly activates caspase-3, and can also cleave Bid to induce the release of cytochrome c (Rousalova and Krepela, 2010). There is also evidence that DCs themselves can acquire killer activity and express granzyme and perforin.

Several strategies are being employed in the field of tumor immunology, including the use of monoclonal antibodies against specific tumor-associated antigens to achieve steric inhibition and neutralization, complement activation, and activation of cell-mediated cytotoxicity, and so-called "cancer vaccines" (Dougan and Dranoff, 2009).

Vaccination against infectious diseases has proven to be one of the great successes of modern medicine, inducing efficient, specific activation of cytotoxic T lymphocytes, as well as the generation of memory cells, protecting against future infection. Translation of this knowledge for the prevention and treatment of cancer has not been straightforward, with, among other things, the selection of appropriate target antigens proving a difficult task, as well as the design and interpretation of clinical trials for this novel class of cancer therapeutics (Lesterhuis, et al., 2011; Palucka, et al., 2011). One example showing great promise with regard to cancer prevention is the development of two human papilloma virus-derived vaccines for the prevention of cervical cancer (Lowy and Schiller, 2006). As for therapeutic vaccination, a series of clinical trials have recently yielded encouraging results. First, treatment of metastatic prostate cancer with sipuleucel-T, a cellular vaccine based on enriched blood DCs briefly cultured with a fusion protein of prostatic acid phosphatase with GM-CSF, resulted in an approximately 4-month–prolonged median survival in phase III trials (Kantoff, et al., 2010). Sipuleucel-T has been approved by the FDA for treatment of metastatic prostate cancer, thereby paving the clinical development and regulatory path for the next generation of cellular immunotherapy products. Second, a

phase III trial in metastatic melanoma testing peptide vaccine in combination with high dose IL-2 versus IL-2 alone showed significant improvement in overall response rate and progression-free survival in patients who received the vaccine (Schwartzentruber, et al., 2011). Third, a phase III trial in patients with follicular lymphoma showed that idiotype vaccine therapy (BiovaxID) significantly prolongs the duration of chemotherapy-induced remission (Morse and Whelan, 2010). Furthermore, a randomized phase II trial of a poxviral-based vaccine targeting a prostate-specific antigen (PROSTVAC) in men with metastatic castration-resistant prostate cancer showed an improved overall survival in patients when compared with patients receiving control vectors (an observed difference in median survival of 8.5 months) (Kantoff, et al., 2010b).

In the sipuleucel-T example described above, DCs are generated *ex vivo*, loaded with tumor antigens, and re-injected to induce strong T-cell and perhaps also natural killer immunity. Another, novel, approach to cancer vaccines is based on the delivery of antigens directly to dendritic cells (DCs) *in vivo*, using chimeric proteins made of anti-DC receptor antibody fused to a selected antigen (DC targeting). Studies in mice demonstrate that DC targeting results in considerable potentiation of antigen-specific T cell immunity. The induction of immunity is observed only when the DC maturation signal is provided, as, otherwise, tolerance ensues (Bonifaz, et al., 2002; Hawiger, et al., 2001). A major challenge of this approach will be to elicit T cell responses that are sufficiently robust and long lasting so as to be clinically active. Indeed, the efficacy of DC targeting *in vivo* needs to be established in clinical trials in patients, and early studies are ongoing.

Besides the molecular make-up of the tumor itself, immunotherapeutic approaches also need to consider that of the tumor micro-environment, and aim to trigger a multi-facetted immune

response involving humoral, cellular, and innate immunity (Poschke, et al., 2011). Recent studies have attempted to relieve the suppression of immune activity in the tumor micro-environment (imposed by the cancer cells) by blocking inhibitory signals using monoclonal antibodies, showing promising results (such as blocking cytotoxic T-lymphocyte antigen-4, CTLA-4, with the monoclonal antibody ipilimumab) (Hoos, et al., 2010). Additional efforts to either directly target MDSC or their suppressive mechanisms should aid the development of successful combination-immunotherapies. Due to their immature nature, a potential way to remove MDSC is to force them to differentiate, for example, by using all-trans retinoic acid (ATRA) or vitamin D3, which promotes myeloid differentiation and has been clinically applied (Lathers, et al., 2004; Mirza, et al., 2006).

Exploiting genomic instability

The genetic instability that is characteristic of cancer cells can be both good and bad for anticancer therapy. Although it seems to provide an Achilles' heel that many conventional therapies exploit, genetic instability can also make eradicating cancer more difficult. Because of the abnormally high mutability of many cancer cells, most malignant tumor cell populations are heterogeneous in many respects, which may make them difficult to target with a single type of treatment. Moreover, this mutability allows many cancers to evolve resistance to therapeutic drugs at an alarming rate (Rajagopalan and Lengauer, 2004).

The novel “synthetic lethal” approach aims to exploit defects in DNA repair pathways using a new theory (Yap, et al., 2011). This theory proposes that targeting tumor cells, genetically defective in one given pathway, with a specific molecular therapy, designed to inhibit a “synthetic lethal” gene partner involved in a complementary pathway, results in selective tumor cell killing. Studies have shown that breast cancer cells, defective in homologous recombination due to BRCA1/2

defects are highly sensitive to blockade of the base excision repair (BER) pathway via inhibition of the poly (ADP-ribose) polymerase (PARP) enzyme, providing strong evidence for the clinical application of this approach (Bryant, et al., 2005; Farmer, et al., 2005). PARP inhibitors might also be used in combination with standard radio- and chemotherapy to sensitize cells to cytotoxic DNA damage; additionally, some PARP inhibitors seem to possess anti-angiogenic activity, making their application in the clinic even more attractive (Mangerich and Bürkle, 2011).

The synthetic lethal concept has also been applied for the treatment of p53-deficient cancers, which, as discussed before, occur rather frequently. Loss of p53 function renders cells dependent on the checkpoint kinase Chk1 for activation of cell cycle checkpoints; hence, inhibition of Chk1 in the presence of DNA damage or replicative stress should lead to mitotic catastrophe and cell death in p53-deficient tumors while sparing normal cells. Several Chk1 inhibitors have now been developed; after showing promising results in preclinical models, they are currently being evaluated in phase I clinical trials (Ma, et al., 2011).

Proteins selectively killing tumor cells

In recent years, a unique set of molecules possessing a remarkable ability have been identified: these proteins are able to detect the malfunctioning of a presumably shared set of cancer-critical genes, and selectively kill the corresponding tumor cells, leaving the normal cells unharmed. Among these proteins killing tumor cells (PKTC) are the human cytokines TRAIL and MDA7/IL24, the frog-derived Brevinin-2R, the human pro-apoptotic Par-4 and Noxa proteins and the organic cation transporter-like 3 (ORCTL3), the alpha-lactalbumin and oleic acid complex HAMLET, and the viral proteins adenovirus E4ORF4, parvovirus NS1, and the Chicken Anemia Virus-encoded protein apoptin (Bruno, et al., 2009; Grimm and Noteborn, 2010;

Argiris, et al., 2011). As these PKTC appear to function independently of the type of cancer, their therapeutic potential seems unprecedented. Apoptin, the first of these proteins to be discovered, and the subject of the present thesis, will be discussed in the next section.

2.6 Apoptin

Apoptin is the protein product of the VP3 gene of the Chicken Anemia Virus (CAV; Noteborn, et al., 1994). The pathogenesis of CAV infection and discovery of apoptin will be discussed in section 2.6.1, while the characteristics of apoptin and perspectives for application as an anti-cancer agent are discussed in sections 2.6.2 and 2.6.3, respectively.

2.6.1 Chicken Anemia Virus

In young chicks, CAV infection results in severe anemia and immunodeficiency (among other symptoms), owing to apoptosis of the cells in the thymus, bone marrow and spleen (Noteborn and Koch, 1995). Early after infection, a 2.3 kb polycistronic mRNA is transcribed, encoding three genes: the capsid protein VP1, the scaffold protein and phosphatase VP2, and VP3 (apoptin) (Noteborn, 2004). Later on in the infection, splicing of the CAV mRNA produces additional RNA products; it is however not known whether these result in functional proteins (Kamada, et al., 2006).

Studies in transformed chicken cells in culture proved that apoptin was responsible for the CAV-induced apoptosis (Noteborn, et al., 1994). Apoptin demonstrated the same apoptosis activity in human cancer cell lines, but, surprisingly, not in normal human cells (Backendorf, et al., 2008; Danen-van Oorschot, et al., 1997; Noteborn, et al., 2005; Tavassoli, et al., 2005). This makes apoptin itself an interesting protein for therapeutic treatment of cancer cells, but also an interesting tool to investigate the process of oncogenic transformation, thereby uncovering other novel targets for anticancer

therapies. Furthermore, apoptin may also prove useful in the diagnosis of cancer, e.g. based on its ability to be specifically phosphorylated by tumor cell lysates (see below).

2.6.2 Characteristics of apoptin-induced apoptosis

Apoptin is a small (121 aa), proline-rich protein, exhibiting little homology to any other known protein – including its functional equivalent TAIP, encoded by the TT virus (Kooistra, et al., 2004). Tumor-selective apoptosis induction by apoptin is preceded by a) phosphorylation at T108 (Rohn, et al., 2002), and b) nuclear translocation (Danen-van Oorschot, et al., 2003) (Figure 2.5). These effects are characteristic for the transformed environment, as they are also elicited upon transient transformation of normal human cells by the SV40 LT and ST proteins (Zhang, et al., 2004).

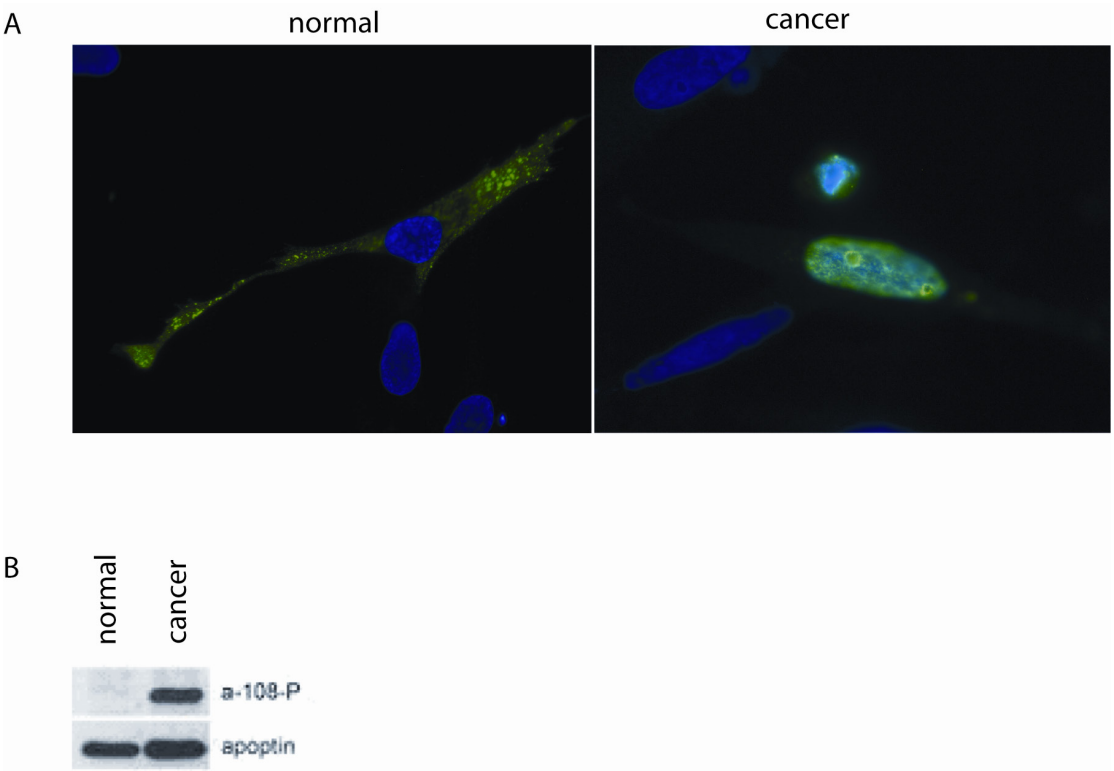

Figure 5. Characteristics of tumor-selective apoptosis induction by apoptin. **A.** In normal cells (left panel), apoptin is located in the cytoplasm, and can be found in granules extending to the cell membrane. In tumor cells (right panel), apoptin is located in the nucleus and induces apoptosis (arrow indicates dying cell). **B.** In tumor cells, but not in normal cells, apoptin is phosphorylated at position T108.

The detailed mechanisms behind both the up- and downstream pathways of apoptin activity in transformed and cancer cells are yet to be elucidated. A number of apoptin-interacting proteins have been identified, including Hippi, the protein interactor and apoptosis co-mediator of the huntingtin interacting protein 1 (Cheng, et al., 2003), and Rybp/DEDAF, a transcription factor and proapoptotic protein known to associate with the death effector domain-containing DNA binding protein DEDD (Danen-van Oorschot, et al., 2004).

Furthermore, apoptin has been shown to act through the intracellular apoptosis pathway, involving the translocation of Nur77 from the nucleus to the cytoplasm, as well as p73 and PUMA activity (Klanrit, et al., 2008; Maddika, et al., 2005). Apoptin expression enhances the level of the tumor suppressor ceramide in tumor cells, also implicating the involvement of sphingolipids in apoptin-induced cell death (Liu, et al., 2006a, b).

2.6.3 Perspectives for apoptin anti-tumor therapy

Though its pathway of action remains to be clarified, apoptin has demonstrated several features that make it well suited for cancer therapy. Besides the fact that it has been shown to selectively kill tumor and transformed cells, apoptin has also been shown to act independently of p53 (Teodoro, et al., 2004; Zhuang, et al., 1995). Furthermore, it is insensitive to BCR-ABL and Bcl-XL (Backendorf, et al., 2008; Noteborn, et al., 2005), which suggests that apoptin might induce apoptosis in cases where other (chemo)therapeutics might fail. The results of the first preclinical therapeutic studies, discussed below, are very promising.

Apoptin cancer therapy using adenoviral vectors

Pietersen and colleagues developed a strategy for the use of apoptin in cancer therapy based on adenoviral vectors (Pietersen, et al., 1999). A single injection of the apoptin-producing adenovirus in xenografted

hepatomas in nude mice resulted in a delay in tumor growth. The number of proliferating cells as detected by BrdU-labeling was dramatically decreased in the apoptin-transduced regions versus control-treated tumors (Van der Eb, et al., 2002). Importantly, the apoptin-producing adenovirus did not have appreciable toxic effects when injected intraperitoneally, intravenously, or subcutaneously into healthy rats. Further studies using these and other non-replicative viruses, including a fowlpox virus–based vector, used a regimen of multiple intratumoral injections during several days. These approaches resulted in a significant overall survival benefit for the apoptin-treated mice and, in some cases, depending on the overall transduction efficiency, complete regression of the established tumor (Noteborn, 2009).

PDT4-apoptin - topical cancer treatment

Guelen and coworkers fused apoptin to the HIV-TAT protein transduction domain, demonstrating efficient transduction of apoptin into both normal and tumor cells, while preserving its tumor-selective apoptotic properties (Guelen, et al., 2004). Sun and colleagues used a similar fusion product, employing protein transduction domain 4 (PTD4)-mediated transduction of recombinant apoptin protein, to treat tumors that had been xenografted onto nude mice (Sun, et al., 2009). In contrast to the gene therapy-based studies discussed above, PTD4-apoptin was administered by simple application onto the epidermis. Remarkably, though the protein could be detected in the epidermal tissue covering the subcutaneous tumor tissue and in several internal organs of the mice, cell death was only observed inside the tumor mass.

Recently, Jin and colleagues (Jin, et al., 2011) reported that PTD4-apoptin protein and dacarbazine acted synergistically to reduce tumor growth in a mouse melanoma model. Importantly, the combination with PTD4-apoptin allowed for a 50% reduction in the dosage of

dacarbazine, resulting in comparable reduction of tumor-growth, without any detectable hematological side-effect.

Systemic apoptin treatment and organ-specific targeting

Peng et al. showed that apoptin can be safely administered systemically, and used a specific ligand to the asialoglycoprotein receptor to target apoptin specifically to the liver (Peng, et al., 2007). Delivery of this Asor-apoptin via the tail vein into mice bearing in situ hepatocarcinomas resulted in specific and efficient distribution of apoptin in both hepatocarcinoma cells and normal liver cells. Whereas the former cells showed significant signs of regression, the normal hepatocytes were clearly not affected.

Specific targeting of apoptin to the brain is currently being developed by de Boer and coworkers. Using the nontoxic ligand CRM197 (a mutant of the diphtheria toxin), the membrane-bound precursor of heparin-binding epidermal growth factor (HB-EGF), also known as the diphtheria toxin receptor (DTR), can be targeted. This receptor is constitutively present at the blood-brain barrier and is strongly up-regulated in many tumors, including human glioblastoma (Mishima, et al., 1998). Biopharmaceutical drugs have been selectively delivered to the brain via this receptor (Gaillard, et al., 2005). An apoptin-expressing plasmid coupled to CRM197 has been successfully delivered to human glioblastoma cells *in vitro* through receptor-mediated endocytosis (Rip, et al., 2009). The combination of apoptin antitumor therapy and CRM197-targeting technology thus provides a great opportunity for development of targeted therapy for brain tumors.

Combination therapy with apoptin and chemo- or radiotherapy

The combination of apoptin therapy with chemotherapeutic agents has been reported to enhance cytotoxicity to human tumor cells *in vitro* (Olijslagers, et al., 2007). Combined treatment of recombinant

adenovirus expressing apoptin with different concentrations of etoposide clearly showed an additive cytotoxic effect in human osteosarcoma U2OS cells. Paclitaxel combined with apoptin acted additively in p53-positive human osteosarcoma U2OS and nonsmall cell lung carcinoma A549 cells, p53-negative osteosarcoma Saos-2 cells, and p53-mutant prostate cancer Du145 cells. Finally, apoptin was proven to be coeffective when combined with the chemotherapeutic agent methotrexate (Zhang, et al., 2007).

Recently, apoptin has also been combined with other treatments *in vivo*. As discussed above, apoptin expression modulates the ceramide-sphingolipid pathways leading to enhanced ceramide levels (Liu, et al., 2006). The majority of prostate tumors have elevated acid ceramidase levels compared with neighboring normal prostate tissue (Liu, et al., 2006). *In vitro*, up-regulated acid-ceramidase protected cells from apoptin-induced apoptosis, whereas cotreatment with the acid-ceramidase inhibitor LCL204 sensitized cells for apoptosis. *In vivo*, combined treatment enhanced the antitumor activity of apoptin in xenografted prostate tumors in mice, resulting in significantly reduced tumor growth and increased animal survival.

Lian and colleagues combined apoptin treatment with interleukin-18 (IL-18) and reported that combined administration results in an even higher induction of an effective antitumor immune response and tumor regression (Lian, et al., 2007). IL-18 and apoptin appear to affect tumors via complementary pathways. Whereas apoptin directly targets the tumor cells, IL-18 treatment appears to act via enhancing the immune response toward tumor cells. Finally, Schoop et al. showed that treatment of a radioresistant head- and neck cancer cell line with apoptin concurrently with exposure to irradiation sensitized these cells to apoptosis (Schoop, et al., 2010).

Tumor-selective targeting using apoptin NLS

Rather than using apoptin itself, or one of its cellular targets, to design anti-cancer therapies, Wagstaff and Jans propose the use of apoptin's tumor-selective NLS (74-121) to target drugs specifically to the nucleus of tumor cells (Wagstaff and Jans, 2009). Delivery of cytotoxic agents directly to the heart of the tumor cell should result in efficient tumor cell killing without affecting healthy neighboring cells.

2.7 Synopsis

The new era of genomics and proteomics carries with it a great promise: that for every disease, the blueprint of the defective cell can be compared with that of a healthy one, thereby not only facilitating the identification of the root of the problem, but also, and more importantly, allow one to charter an efficient route to fix it. Drawing up the blueprint of a cancer cell has proven to be a difficult task. Even so, a complex map of intracellular pathways is starting to emerge, with a number of essential traits being seemingly shared by all transformed cells. The CAV-derived apoptin apparently senses these characteristics and effectively charters its own route to selectively kill malignant cells. The properties of apoptin and functionally related proteins, combined with newly developed therapeutics targeting the hallmark capabilities and enabling characteristics of cancer cells, should finally lead to the development of selective, efficient and robust therapies for cancer – instead of the long sought-after "magical bullet" cure for cancer, we will be able to build a magical cluster bomb.

References

Al-Hajj M, Wicha M, Benito-Hernandez A, Morrison S and Clarke, M. (2003) Prospective identification of tumorigenic breast cancer cells. *PNAS* 100, 3983-3988.

Alexandre J, Batteux F, Nicco C, Chéreau C, Laurent A, Guillevin L et al. (2006) Accumulation of hydrogen peroxide is an early and crucial step for paclitaxel-induced cancer cell death both in vitro and in vivo. *Int J Cancer* 119, 41-48.

Ali S and DeCaprio J (2001) Cellular transformation by SV40 large T antigen: interaction with host proteins. *Semin Cancer Biol* 11, 15-23.

Amaravadi R, Yu D, Lum J, Bui T, Christophorou M, Evan G et al. (2007) Autophagy inhibition enhances therapy-induced apoptosis in a myc-induced model of lymphoma . *J Clin Invest 117,* 326-336.

Apel A, Zentgraf H, Buchler M and Herr I (2009) Autophagy-a double edged sword in oncology. *Int J Cancer* 125, 991-995.

Argiris K, Panethymitaki C and Tavassoli M (2011) Naturally occurring, tumor-specific, therapeutic proteins. *Exp Biol Med* 236, 524-536.

Assouline S, Culjkovic B, Cocolakis E, Rousseau C, Beslu N, Amri A et al. (2009) Molecular targeting of the oncogene eIF4E in acute myeloid leukemia (AML): a proof-of-principle clinical trial with ribavirin. *Blood* 114, 257–260.

Backendorf C, Visser AE, De Boer AG, Zimmerman R, Visser M, Voskamp P, Zhang Y-H and Noteborn MHM (2008) Apoptin: therapeutic potential of an early sensor of carcinogenic transformation. *Annu Rev Pharmacol Toxicol* 48, 143-169.

Baehrecke E (2003) Autophagic programmed cell death in Drosophila. *Cell Death Differ* 10, 940-945.

Bergers G and Benjamin L (2003) Tumorigenesis and the angiogenic switch. *Nat Rev Cancer 3*, 401-410.

Berx G and van Roy F (2009) Involvement of members of the cadherin superfamily in cancer. *Cold Spring Harb Perspect Biol* 1, a003129.

Bhowmick N, Neilson E and Moses H (2004) Stromal fibroblasts in cancer initiation and progression. *Nature* 432, 332-337.

Bindea G, Mlecnik B, Fridman W, Pagès F and Galon J (2010) Natural immunity to cancer in humans. *Curr Opin Immunol* 22, 215-222.

Blackburn E (2000) Telomere states and cell fates. *Nature* 408, 53-56.

Blaschuk O and Devemy E (2009) Cadherins as novel targets for anti-cancer therapy. *Eur J Pharmacol* 625, 195-198.

Blomen V and Boonstra J (2007) Cell fate determination during G1 phase progression. *Cell Mol Life Sci* 64, 3084-3104.

Bonifaz L, Bonnyay D, Mahnke K, Rivera M, Nussenzweig M and Steinman R (2002) Efficient targeting of protein antigen to the dendritic cell receptor DEC-205 in the steady state leads to antigen presentation on major histocompatibility complex class I products and peripheral CD8+ T cell tolerance. *J Exp Med* 196, 1627–1638.

Bonnet S, Archer S, Allalunis-Turner J, Haromy A, Beaulieu C, Thompson R et al. (2007) A mitochondria-K+ channel axis is suppressed in cancer and its normalization promotes apoptosis and inhibits cancer growth. *Cancer Cell* 11, 37-51.

Boya P, González-Polo R, Casares N, Perfettini J, Dessen P, Larochette N et al. (2005) Inhibition of macroautophagy triggers apoptosis. *Mol Cell Biol* 25, 1025-1040.

Brosh R and Rotter V (2009) When mutants gain new powers: news from the mutant p53 field. *Nat Rev Cancer* 9, 701-713.

Bruno P, Brinkmann C, Boulanger M, Flinterman M, Klanrit P, Landry M et al. (2009) Family at last: highlights of the first international meeting on proteins killing tumour cells. *Cell Death and Differentiation* 16, 184-186.

Bryan T, Englezou A, Dalla-Pozza L, Dunham M and Reddel R (1997) Evidence for an alternative mechanism for maintaining telomere length in human tumors and tumor-derived cell lines. *Nat Med* 3, 1271-1274.

Bryan T, Englezou A, Dunham M and Reddel R (1998) Telomere length dynamics in telomerase-positive immortal human cell populations. *Exp Cell Res* 239, 370–378.

Bryant H, Schultz N, Thomas H, KM P, Flower D, Lopez E et al. (2005) Specific killing of BRCA2-deficient tumours with inhibitors of poly(ADP-ribose) polymerase. *Nature* 434, 913-917.

Campisi J and d'Adda di Fagagna F (2007) Cellular senescence: when bad things happen to good cells. *Nat Rev Mol Cell Biol* 8, 729-740.

Carmeliet P (2005) VEGF as a key mediator of angiogenesis in cancer. *Oncology* 69, 4-10.

Castedo M, Perfettini J, Roumier T, Andreau K, Medema R and Kroemer G (2004) Cell death by mitotic catastrophe: a molecular definition. *Oncogene* 23, 2825-2837.

Chang H (2010) Trastuzumab-based neoadjuvant therapy in patients with HER2-positive breast cancer. *Cancer* 116, 2856-2867.

Chen N and Karantza V (2011) Autophagy as a therapeutic target in cancer. *Cancer Biol Ther* 11, 157-168.

Chen Q, Espey M, Krishna M, Mitchell J, Corpe C, Buettner G et al. (2005) Pharmacologic ascorbic acid concentrations selectively kill cancer cells: action as a pro-drug to deliver hydrogen peroxide to tissues. *Proc Natl Acad Sci U S A* 102, 13604-13609.

Chen Z, Lu W, Garcia-Prieto C and Huang P (2007) The Warburg effect and its cancer therapeutic implications. *J Bioenerg Biomembr* 39, 267–274.

Cheng C, Huang S, Chang Y, Chung W and Yuo C (2003) The viral death protein Apoptin interacts with Hippi, the protein interactor of Huntingtin-interacting protein 1. *Biochem Biophys Res Commun* 305, 359-364 .

Cheng N, Chytil A, Shyr Y, Joly A and Moses H (2008) Transforming growth factor-beta signaling-deficient fibroblasts enhance hepatocyte growth factor signaling in mammary carcinoma cells to promote scattering and invasion. *Mol Cancer Res* 6, 1521-1533.

Cho R and Clarke M (2008) Recent advances in cancer stem cells. *Curr Opin Genet Dev* 18, 48-53.

Chonghaile T and Letai A (2008) Mimicking the BH3 domain to kill cancer cells. *Oncogene* 27, S149-S1457.

Christoffersen T, Guren T, Spindler K, Dahl O, Lønning P and Gjertsen B (2009) Cancer therapy targeted at cellular signal transduction mechanisms: strategies, clinical results, and unresolved issues. *Eur J Pharmacol* 625, 6-22.

Cleveland D, Mao Y and Sullivan K (2003) Centromeres and kinetochores: from epigenetics to mitotic checkpoint signaling. *Cell* 112, 407-421.

Colanzi A and Corda D (2007) Mitosis controls the Golgi and the Golgi controls mitosis. *Curr Opin Cell Biol* 19, 386-393.

Cook K and Figg W (2010) Angiogenesis inhibitors: current strategies and future prospects. *CA Cancer J Clin* 60, 222-243.

Counter C, Hirte H, Bacchetti S and Harley C (1994) Telomerase activity in human ovarian carcinoma. *Proc Natl Acad Sci U S A* 91, 2900-2904.

d'Adda di Fagagna F, Reaper P, Clay-Farrace L, Fiegler H, Carr P, Von Zglinicki T et al. (2003) A DNA damage checkpoint response in telomere-initiated senescence. *Nature* 426, 194-198.

Danen-van Oorschot A, Fischer D, Grimbergen J, Klein B, Zhuang S-M, Falkenburg J et al. (1997) Apoptin induces apoptosis in human transformed and malignant cells but not in normal cells. *Proc. Natl. Acad. Sci. U.S.A.* 94, 5843-5847.

Danen-van Oorschot A, Voskamp P, Seelen M, van Miltenburg M, Bolk M, Tait S et al. (2004) Human death effector domain-associated factor interacts with the viral apoptosis agonist Apoptin and exerts tumor-preferential cell killing. *Cell Death and Differentiation* 11, 564-573.

Danen-van Oorschot A, Zhang Y-H, Leliveld S, Rohn J, Seelen M, Bolk M et al. (2003) Importance of nuclear localization of apoptin for tumor-specific induction of apotosis. *J Biol Chem* 278, 27729-27736.

Dang C and Semenza G (1999) Oncogenic alterations of metabolism. *Trends Biochem Sci* 24, 68-72.

Darwin C (1859) *On the Origin of Species by Means of Natural Selection, or the Preservation of Favoured Races in the Struggle for Life.* London: John Murray.

Davies H (2002) Mutations of the BRAF gene in human cancer. *Nature* 417, 949–954.

de Bruin E and Medema J (2008) Apoptosis and non-apoptotic deaths in cancer development and treatment response. *Biochem Pharmacol* 76, 947-957.

De Falco M and De Luca A (2010) Cell cycle as a target of antineoplastic drugs. *Curr Pharm Des* 16, 1417-1426.

DeBerardinis R, Lum J, Hatzivassiliou G and Thompson C (2008) The biology of cancer: metabolic reprogramming fuels cell growth and proliferation. *Cell Metab* 7, 11-20.

Desgrosellier J and Cheresh D (2010) Integrins in cancer: biological implications and therapeutic opportunities. *Nat Rev Cancer* 10, 9-22.

Dougan M and Dranoff G (2009) Immune therapy for cancer. *Annu Rev Immunol* 27, 83-117.

Downward J (2003) Targeting RAS signalling pathways in cancer therapy. *Nat Rev Cancer* 3, 11-22.

Drygin D, Rice W and Grummt I (2010) The RNA polymerase I transcription machinery: an emerging target for the treatment of cancer. *Annu Rev Pharmacol Toxicol* 50, 131-156.

Drygin D, Siddiqui-Jain A, O'Brien S, Schwaebe M, Lin A, Bliesath J et al. (2009) Anticancer activity of CX-3543: a direct inhibitor of rRNA biogenesis. *Cancer Res* 69, 7653-7661.

Dufner A and Thomas G (1999) Ribosomal S6 kinase signaling and the control of translation. *Exp Cell Res* 253, 100-109.

Elstrom R, Bauer D, Buzzai M, Karnauskas R, Harris M, Plas D et al. (2004) Akt stimulates aerobic glycolysis in cancer cells. *Cancer Res* 64, 3892–3899.

Farmer H, McCabe N, Lord C, Tutt A, Johnson D, Richardson T et al. (2005) Targeting the DNA repair defect in BRCA mutant cells as a therapeutic strategy. *Nature* 434, 917-921.

Ferrara N (2009) Vascular endothelial growth factor. *Arterioscler Thromb Vasc Biol* 29, 789–791.

Festjens N, Vanden Berghe T and Vandenabeele P (2006) Necrosis, a well-orchestrated form of cell demise: signalling cascades, important mediators and concomitant immune response. *Biochim Biophys Acta* 1757, 1371–1387.

Festjens N, Vanden Berghe T, Cornelis S and Vandenabeele P (2007) RIP1, a kinase on the crossroads of a cell's decision to live or die. *Cell Death Differ* 14, 400-410.

Foley E, Maldonado M, and Kapoor T (2011) Formation of stable attachments between kinetochores and microtubules depends on the B56-PP2A phosphatase. *Nat Cell Biol,* 13, 1265-1271.

Forester C, Maddox J, Louis J, Goris J and Virshup D (2007) Control of mitotic exit by PP2A regulation of Cdc25C and Cdk1. *Proc Natl Acad Sci USA* 104, 19867-19872.

Freudenberg J, Wang Q, Katsumata M, Drebin J, Nagatomo I and Greene M (2009) The role of HER2 in early breast cancer metastasis and the origins of resistance to HER2-targeted therapies. *Exp Mol Pathol 87*, 1-11.

Friedl P and Wolf K (2008) Tube travel: the role of proteases in individual and collective cancer cell invasion. *Cancer Res* 68, 7247-7249.

Gaillard P, Brink A and de Boer A (2005) Diphtheria toxin receptor-targeted brain drug delivery. *Int Congr Ser* 1277, 185–195.

Galluzzi L, Vitale I, Abrams J, Alnemri E, Baehrecke E, Blagosklonny M et al. (2011) Molecular definitions of cell death subroutines: recommendations of the Nomenclature Committee on Cell Death 2012. *Cell Death Differ Epub ahead of print.*

Gatenby R and Gillies R (2004) Why do cancers have high aerobic glycolysis? *Nat Rev Cancer* 4, 891-899.

Gatenby R and Gillies R (2007) Glycolysis in cancer: a potential target for therapy. *Int J Biochem Cell Biol* 39, 1358–1366.

Gewirtz D, Holt S and Elmore L (2008) Accelerated senescence: an emerging role in tumor cell response to chemotherapy and radiation. *Biochem Pharmacol* 76, 947-957.

Ghoshal K and Jacob S (1997) An alternative molecular mechanism of action of 5-fluorouracil, a potent anticancer drug. *Biochem Pharmacol* 53, 1569–1575.

Ghotra V, Puigvert J and Danen E (2009) The cancer stem cell microenvironment and anti-cancer therapy. *Int J Radiat Biol* 85, 955-692.

Gingras A, Raught B and Sonenberg N (2004) mTOR signaling to translation. *Curr Top Microbiol Immunol* 279, 169-197.

Grimm S and Noteborn M (2010) Anti-cancer genes: inducers of tumour-specific cell death signalling. *Trends in Molecular Medicine* 16, 88-96.

Guelen L, Paterson H, Gäken J, Meyers M, Farzaneh F and Tavassoli M (2004) TAT-apoptin is efficiently delivered and induces apoptosis in cancer cells. *Oncogene* 23, 1153-1165.

Gyrd-Hansen M and Meier P (2010) IAPs: from caspase inhibitors to modulators of NF-kappaB, inflammation and cancer. *Nat Rev Cancer* 10, 561-574.

Hahn W and Weinberg R (2002) Modeling the molecular circuitry of cancer. *Nat Rev Cancer* 331, 331-341.

Hahn W and Weinberg R (2002b) Rules for making human tumor cells. *N Engl J Med* 347, 1593-1603.

Hanahan D and Weinberg R (2000) The Hallmarks of cancer. *Cell* 100, 57-70.

Hanahan D and Weinberg R (2011) Hallmarks of cancer: the next generation. *Cell* 144, 646-674.

Hara E, Tsurui H, Shinozaki A, Nakada S and Oda K (1991) Cooperative effect of antisense-Rb and antisense-p53 oligomers on the

extension of life span in human diploid fibroblasts, TIG-1. *Biochem Biophys Res Commun* 179, 528-534.

Harley C, Futcher A and Greider C (1990) Telomeres shorten during ageing of human fibroblasts. *Nature* 345, 458-460.

Hawiger D, Inaba K, Dorsett Y, Guo M, Mahnke K, Rivera M et al. (2001) Dendritic cells induce peripheral T cell unresponsiveness under steady state conditions in vivo. *J Exp Med* 194, 769–779.

Hayflick L (1965) The limited in vitro lifetime of human diploid cell strains. *Exp Cell Res* 37, 614-636.

Hayflick L and Moorhead P (1961) The serial cultivation of human diploid cell strains. *Exp Cell Res* 25, 585-621.

Heichman K and Roberts J (1994) Rules to replicate by. *Cell* 79, 557-562.

Heit R, Rattner J, Chan G and Hendzel M (2009) G2 histone methylation is required for the proper segregation of chromosomes. *J Cell Sci* 122, 2957-2968.

Hezel A and Bardeesy N (2008) LKB1; linking cell structure and tumor suppression. *Oncogene* 27, 6908-6919.

Hoos A, Ibrahim R, Korman A, Abdallah K, Berman D, Shahabi V et al. (2010) Development of ipilimumab: contribution to a new paradigm for cancer immunotherapy. *Semin Oncol* 37, 533-546.

Hunter A, LaCasse E and Korneluk R (2007) The inhibitors of apoptosis (IAPs) as cancer targets. *Apoptosis* 12, 1543-1568.

Jiang B and Liu L (2009) PI3K/PTEN signaling in angiogenesis and tumorigenesis. *Adv Cancer Res* 102, 19-65.

Jin J, Gong J, Yin T, Lu Y, Xia J, Xie Y et al. (2011) PTD4-apoptin protein and dacarbazine show a synergistic antitumor effect on B16-F1 melanoma in vitro and in vivo. *Eur J Pharmacol* 654, 17-25.

Jin S and White E (2007) Role of Autophagy in Cancer. *Autophagy* 3, 28-31.

Jing Y, Dai J, Chalmers-Redman R, Tatton W and Waxman S. (1999) Arsenic trioxide selectively induces acute promyelocytic leukemia cell apoptosis via a hydrogen peroxide-dependent pathway. *Blood 94*, 2102–2111.

Jones W (1868) *Hippocrates Collected Works I.* Cambridge: Harvard University Press.

Jordan P and Carmo-Fonseca M (1998) Cisplatin inhibits synthesis of rRNA in vivo. *Nucleic Acids Res* 26, 2831–2836.

Kalyankrishna S and Grandis J (2006) Epidermal growth factor receptor biology in head and neck cancer. *J Clin Oncol* 24, 2666-2672.

Kamada K, Kuroishi A, Kamahora T, Kabat P, Yamaguchi S and Hino S (2006) Spliced mRNAs detected during the life cycle of chicken anemia virus. *J Gen Virol* 87, 2227–2233.

Kang M and Reynolds C (2009) Bcl-2 inhibitors: targeting mitochondrial apoptotic pathways in cancer therapy. *Clin Cancer Res* 15, 1126-1132.

Kantoff P, Higano C, Shore N, Berger E, Small E, Penson D et al. (2010) Sipuleucel-T immunotherapy for castration-resistant prostate cancer. *N Engl J Med* 363, 411-422.

Kantoff P, Schuetz T, Blumenstein B, Glode L, Bilhartz D, Wyand M et al. (2010b) Overall survival analysis of a phase II randomized controlled trial of a Poxviral-based PSA-targeted immunotherapy in metastatic castration-resistant prostate cancer. *J Clin Oncol* 28, 1099-1105.

Karantza-Wadsworth V, Patel S, Kravchuk O, Chen G, Mathew R, Jin S, et al. (2007) Autophagy mitigates metabolic stress and genome damage in mammary tumorigenesis. *Genes Dev* 21, 1621-1635.

Karnoub A and Weinberg R (2008). Ras oncogenes: split personalities. *Nat Rev Mol Cell Biol* 9, 517-531.

Kelland L (2005) Overcoming the immortality of tumour cells by telomere and telomerase based cancer therapeutics--current status and future prospects. *Eur J Cancer* 41, 971-979.

Kelland L (2007). Targeting the limitless replicative potential of cancer: the telomerase/telomere pathway. *Clin Cancer Res* 13, 4960-4963.

Kessenbrock K, Plaks V and Werb Z (2010) Matrix metalloproteinases: regulators of the tumor microenvironment. *Cell* 141, 52-67.

Kim N, Piatyszek M, Prowse K, Harley C, West M, Ho P et al. (1994) Specific association of human telomerase activity with immortal cells and cancer. *Science* 266, 2011-2015.

Kim R, Emi M and Tanabe K (2007) Cancer immunoediting from immune surveillance to immune escape. *Immunology* 121, 1–14.

Klanrit P, Flinterman M, Odell E, Melino G, Killick R, Norris J et al. (2008) Specific isoforms of p73 control the induction of cell death induced by the viral proteins, E1A or apoptin. *Cell Cycle* 7, 205-215.

Klionsky D and Emr S (2000) Autophagy as a regulated pathway of cellular degradation. *Science* 290, 1717-1721.

Knudson AJ (1971) Mutation and cancer: statistical study of retinoblastoma. *Proc Natl Acad Sci U S A* 68, 820-823.

Kooistra K, Zhang Y-H, Henriquez N, Weiss B, Mumberg D and Noteborn M (2004) TT virus-derived apoptosis-inducing protein induces apoptosis preferentially in hepatocellular carcinoma-derived cells. *J Gen Virol* 85, 1445-1450.

Kroemer G, Galluzzi L, Vandenabeele P, Abrams J, Alnemri E, Baehrecke E et al. (2009) Classification of cell death:

recommendations of the Nomenclature Committee on Cell Death 2009. *Cell Death Differ* 16, 3-11.

Kurokawa M and Kornbluth S (2009) Caspases and kinases in a death grip. *Cell* 138, 838-854.

LaCasse E, Mahoney D, Cheung H, Plenchette S and Korneluk, R. (2008) IAP-targeted therapies for cancer. *Oncogene* 27, 6252-6275.

Lassus P, Opitz-Araya X and Lazebnik Y (2002) Requirement for caspase-2 in stress-induced apoptosis before mitochondrial permeabilization. *Science* 297, 1352-1354.

Lathers D, Clark J, Achille N and Young M (2004) Phase 1B study to improve immune responses in head and neck cancer patients using escalating doses of 25-hydroxyvitamin D3. *Cancer Immunol Immunother* 53, 422–430.

Lengauer C, Kinzler K and Vogelstein B (1998) Genetic instabilities in human cancers. *Nature* 396, 643-649.

Lesterhuis W, Haanen J and Punt C (2011) Cancer immunotherapy-revisited. *Nat Rev Drug Discov* 10, 591-600.

Levy L and Hill C (2006) Alterations in components of the TGF-beta superfamily signaling pathways in human cancer. *Cytokine Growth Factor Rev* 17, 41-58.

Lian H, Jin N, Li X, Mi Z, Zhang J, Sun L et al. (2007) Induction of an effective anti-tumor immune response and tumor regression by combined administration of IL-18 and Apoptin. *Cancer Immunol Immunother* 56, 181-192.

Liu X, Elojeimy S, El-Zawahry A, Holman D, Bielawska A, Bielawski J et al. (2006) Modulation of ceramide metabolism enhances viral protein apoptin's cytotoxicity in prostate cancer. *Mol Ther* 14, 637-646.

Liu X, Zeidan Y, Elojeimy S, Holman D, El-Zawahry A, Guo G et al. (2006) Involvement of sphingolipids in apoptin-induced cell killing. *Mol Ther* 14, 627–636.

Lobo N, Shimono Y, Qian D and Clarke M (2007) The biology of cancer stem cells. *Annu Rev Cell Dev Biol* 23, 675-699.

Lock R and Stribinskiene L (1996) Dual modes of death induced by etoposide in human epithelial tumor cells allow Bcl-2 to inhibit apoptosis without affecting clonogenic survival. *Cancer Res* 56, 4006-4012.

Lodish H, Berk A, Zipursky SL, Matsudaira P, Baltimore D and Darnell JE (1999) *Molecular Cell Biology.* New York: W. H. Freeman and Co.

Lombaerts M, van Wezel T, Philippo K, Dierssen J, Zimmerman R, Oosting J et al. (2006) E-cadherin transcriptional downregulation by promoter methylation but not mutation is related to epithelial-to-mesenchymal transition in breast cancer cell lines. *Br J Cancer* 94, 661-671.

López-Lázaro M (2008) The Warburg effect: why and how do cancer cells activate glycolysis in the presence of oxygen? *Anticancer Agents Med Chem* 8, 305–312.

López-Lázaro M (2010) A new view of carcinogenesis and an alternative approach to cancer therapy. *Mol Med* 16, 144-153.

Lowy D and Schiller J (2006) Prophylactic human papillomavirus vaccines. *J Clin Invest* 116, 1167-1173.

Lum J, Bauer D, Kong M, Harris M, Li C, Lindsten T et al. (2005) Growth factor regulation of autophagy and cell survival in the absence of apoptosis. *Cell* 120, 237-248.

Ma C, Janetka J and Piwnica-Worms H (2011) Death by releasing the breaks: CHK1 inhibitors as cancer therapeutics. *Trends Mol Med* 17, 88-96.

Mac Gabhann F and Popel A (2008) Systems biology of vascular endothelial growth factors. *Microcirculation* 15, 715-738.

Maddika S, Booy E, Johar D, Gibson S, Ghavami S and Los M (2005) Cancer-specific toxicity of apoptin is independent of death receptors but involves the loss of mitochondrial membrane potential and the release of mitochondrial cell-death mediators by a Nur77-dependent pathway. *J Cell Sci* 118, 4485–4493.

Madsen C and Sahai E (2010) Cancer dissemination--lessons from leukocytes. *Dev Cell* 19, 13-26.

Maiuri M, Zalckvar E, Kimchi A and Kroemer G (2007) Self-eating and self-killing: crosstalk between autophagy and apoptosis. *Nat Rev Mol Cell Biol* 8, 741-752.

Majumder P, Febbo P, Bikoff R, Berger R, Xue Q, McMahon L et al. (2004) mTOR inhibition reverses Akt-dependent prostate intraepithelial neoplasia through regulation of apoptotic and HIF-1-dependent pathways. *Nat Med* 10, 594-601.

Mangerich A and Bürkle A (2011) How to kill tumor cells with inhibitors of poly(ADP-ribosyl)ation. *Int J Cancer* 128, 251-265.

Mani S, Guo W, Liao M, Eaton E, Ayyanan A, Zhou A et al. (2008) The epithelial-mesenchymal transition generates cells with properties of stem cells. *Cell* 133, 704–715.

Marian C, Cho S, McEllin B, Maher E, Hatanpaa K, Madden C et al. (2010) The telomerase antagonist, imetelstat, efficiently targets glioblastoma tumor-initiating cells leading to decreased proliferation and tumor growth. *Clin Cancer Res* 16, 154-163.

Massagué J (2004) G1 cell-cycle control and cancer. *Nature* 432, 298-306.

Mathew R, Karantza-Wadsworth V and White E (2007) Role of autophagy in cancer. *Nat Rev Cancer* 7, 961-967.

Mathew R, Kongara S, Beaudoin B, Karp C, Bray K, Degenhardt K et al. (2007) Autophagy suppresses tumor progression by limiting chromosomal instability. *Genes Dev* 21, 1367-1381.

Mehlen P and Bredesen D (2011). Dependence receptors: from basic research to drug development. *Sci Signal* 4, mr2.

Micalizzi D, Farabaugh S and Ford H (2010) Epithelial-mesenchymal transition in cancer: parallels between normal development and tumor progression. *J Mammary Gland Biol Neoplasia* 15, 117-134.

Mirza N, Fishman M, Fricke I, Dunn M, Neuger A, Frost T et al. (2006) All-trans-retinoic acid improves differentiation of myeloid cells and immune response in cancer patients. *Cancer Res* 66, 9299–9307.

Mishima K, Higashiyama S, Asai A, Yamaoka K, Nagashima Y, Taniguchi N et al. (1998) Heparin-binding epidermal growth factor-like growth factor stimulates mitogenic signaling and is highly expressed in human malignant gliomas. *Acta Neuropathol* 96, 322-328.

Morrish T and Greider C (2009) Short telomeres initiate telomere recombination in primary and tumor cells. *PLoS Genet* 5, e1000357.

Morse M and Whelan M (2010) A year of successful cancer vaccines points to a path forward. *Curr Opin Mol Ther* 12, 11-13.

Mougiakakos D, Choudhury A, Lladser A, Kiessling R and Johansson C (2010) Regulatory T cells in cancer. *Adv Cancer Res* 107, 57-117.

Mumby M (2007) PP2A: unveiling a reluctant tumor suppressor. *Cell* 130, 21-24.

Nardella C, Clohessy J, Alimonti A and Pandolfi P (2011) Pro-senescence therapy for cancer treatment. *Nat Rev Cancer* 11, 503-511.

Narita M, Nũnez S, Heard E, Narita M, Lin A, Hearn S et al. (2003) Rb-mediated heterochromatin formation and silencing of E2F target genes during cellular senescence. *Cell* 113, 703-716.

Nieto M (2011) The ins and outs of the epithelial to mesenchymal transition in health and disease. *Annu Rev Cell Dev Bio* 27, 347-376.

Noteborn M (2004) Chicken anemia virus induced apoptosis: underlying molecular mechanisms. *Vet Microbiol* 98, 89-94.

Noteborn M (2005) Apoptin acts as a tumor-specific killer: potentials for an antitumor therapy. *Cell Mol Biol* 51, 49–60.

Noteborn M (2009) Proteins selectively killing tumor cells. *Eur J Pharmacol* 625, 165-173.

Noteborn M and Koch G (1995) Chicken anaemia virus infection: molecular basis of pathogenicity. *Avian Pathol* 24, 11-31.

Noteborn M, Todd D, Verschueren C, de Gauw H, Curran W, Veldkamp S et al. (1994) A single chicken anemia virus protein induces apoptosis. *J Virol* 68, 346-351.

Olijslagers S, Zhang Y, Backendorf C and Noteborn M (2007) Additive cytotoxic effect of apoptin and chemotherapeutic agents paclitaxel and etoposide on human tumour cells. *Basic Clin Pharmacol Toxicol* 100, 127–131.

Ostman A and Heldin C (2007) PDGF receptors as targets in tumor treatment. *Adv Cancer Res* 97, 247-274.

Ostrand-Rosenberg S and Sinha P (2009) Myeloid-derived suppressor cells: linking inflammation and cancer. *J Immunol* 182, 4499-4506.

Palucka K, Ueno H and Banchereau J (2011) Recent developments in cancer vaccines. *J Immunol* 186, 1325-1331.

Pardo J, Bosque A, Brehm R, Wallich R, Naval J, Müllbacher A et al. (2004) Apoptotic pathways are selectively activated by granzyme A and/or granzyme B in CTL-mediated target cell lysis. *J Cell Biol* 167, 457-468.

Parkinson E and Minty F (2007) Anticancer therapy targeting telomeres and telomerase: current status. *BioDrugs* 21, 375-385.

Paroni G, Henderson C, Schneider C and Brancolini C (2002) Caspase-2 can trigger cytochrome C release and apoptosis from the nucleus. *J Biol Chem* 277, 15147-15161.

Partanen J, Nieminen A and Klefstrom J (2009) 3D view to tumor suppression: Lkb1, polarity and the arrest of oncogenic c-Myc. *Cell Cycle* 8, 716-724.

Peart M, Tainton K, Ruefli A, Dear A, Sedelies K, O'Reilly L et al. (2003). Novel mechanisms of apoptosis induced by histone deacetylase inhibitors. *Cancer Res* 63, 4460-4471.

Pelicano H, Martin D, Xu R and Huang P (2006) Glycolysis inhibition for anticancer treatment. *Oncogene* 25, 4633–4646.

Peng D, Sun J, Wang Y, Tian J, Zhang Y, Noteborn M et al. (2007) Inhibition of hepatocarcinoma by systemic delivery of Apoptin gene via the hepatic asialoglycoprotein receptor. *Cancer Gene Ther* 14, 66-73.

Petiot A, Ogier-Denis E, Blommaart E, Meijer A and Codogno P (2000) Distinct classes of phosphatidylinositol 3'-kinases are involved in signaling pathways that control macroautophagy in HT-29 cells. *J Biol Chem* 275, 992-998.

Pfeiffer T, Schuster S and Bonhoeffer S (2001) Cooperation and compe- tition in the evolution of ATP-producing pathways. *Science* 292, 504–507.

Phatak P and Burger A (2007) Telomerase and its potential for therapeutic intervention. *Br J Pharmacol* 152, 1003-1011.

Pietersen A, van der Eb M, Rademaker H, van den Wollenberg D, Rabelink, M, Kuppen P et al. (1999) Specific tumor-cell killing with adenovirus vectors containing the apoptin gene. *Gene Ther* 6, 882–892.

Pitti RM, Marsters SA, Lawrence DA, Roy M, Kischkel FC, Dowd P et al. (1998) Genomic amplification of a decoy receptor for Fas ligand in lung and colon cancer. *Nature* 396, 699-703.

Poschke I, Mougiakakos D and Kiessling R (2011) Camouflage and sabotage: tumor escape from the immune system. *Cancer Immunol Immunother* 60, 1161-1171.

Qian B-Z and Pollard J (2010) Macrophage Diversity Enhances Tumor Progression and Metastasis. *Cell* 141, 39-51.

Rajagopalan H and Lengauer C (2004) Aneuploidy and cancer. *Nature* 432, 338-341.

Rathmell J, Fox C, Plas D, Hammerman P, Cinalli R and Thompson C (2003) Akt-directed glucose metabolism can prevent Bax conformation change and promote growth factor-independent survival. *Mol Cell Biol* 23, 7315–7328.

Read S, Baliga B, Ekert P, Vaux D and Kumar S (2002) A novel Apaf-1-independent putative caspase-2 activation complex. *J Cell Biol* 159, 739-745.

Reed J (2008) Bcl-2-family proteins and hematologic malignancies: history and future prospects. *Blood* 111, 3322–3330.

Rip J, de Groot A, Voskamp P, Gaillard P, Noteborn M and de Boer A (2009) CRM197-targeted gene delivery to brain tumors. In *Proteins Killing Tumour Cells* (pp. 245-57).

Robertson J, Enoksson M, Suomela M, Zhivotovsky B and Orrenius S (2002) Caspase-2 acts upstream of mitochondria to promote

cytochrome c release during etoposide-induced apoptosis. *J Biol Chem* 277, 29803-29809.

Rohn J, Zhang Y-H, Aalbers R, Otto N, den Hertog J, Henriquez N et al. (2002) A tumor-specific kinase activity regulates the viral death protein apoptin. *J Biol Chem* 277, 50820-50827.

Rosenfeldt M and Ryan K (2009) The role of autophagy in tumour development and cancer therapy. *Expert Rev Mol Med* 11, e36.

Rousalova I and Krepela E (2010) Granzyme B-induced apoptosis in cancer cells and its regulation. *Int J Oncol* 37, 1361-1378.

Roussos E, Condeelis J and Patsialou A (2011) Chemotaxis in cancer. *Nat Rev Cancer* 11, 573-587.

Samassekou O, Gadji M, Drouin R and Yan J (2010) Sizing the ends: Normal length of human telomeres. *Ann Anat,* Epub ahead of print.

Schmalhofer O, Brabletz S and Brabletz T (2009) E-cadherin, beta-catenin, and ZEB1 in malignant progression of cancer. *Cancer Metastasis Rev* 28, 151-166.

Schoop R, Verdegaal E, de Jong R and Noteborn M (2010) Apoptin enhances radiation-induced cell death in poorly responding head and neck squamous cell carcinoma cells. *Basic Clin Pharmacol Toxicol* 106, 130-134.

Schwartzentruber D, Lawson D, Richards J, Conry R, Miller D, Treisman J et al. (2011) gp100 peptide vaccine and interleukin-2 in patients with advanced melanoma. *N Engl J Med 364*, 2119-2127.

Seliger B (2008) Molecular mechanisms of MHC class I abnormalities and APM components in human tumors. *Cancer Immunol Immunother* 57, 1719-1726.

Semenza G (2000) HIF-1 and human disease: one highly involved factor. *Genes Dev* 14, 1983-1991.

Semenza G (2007) HIF-1 mediates the Warburg effect in clear cell renal carcinoma. *J Bioenerg Biomembr* 39, 231-234.

Shaw R (2009) Tumor suppression by LKB1: SIK-ness prevents metastasis. *Sci Signal* 2, pe55.

Shay J and Bacchetti S (1997) A survey of telomerase activity in human cancer. *Eur J Cancer* 33, 787-791.

Shay J, Pereira-Smith O and Wright W (1991) A role for both RB and p53 in the regulation of human cellular senescence. *Exp Cell Res* 196, 33-39.

Sherr C (1996) Cancer cell cycles. *Science* 274, 1672-1677.

Sherr C (2001) The INK4a/ARFnetwork in tumour suppression. *Nat Rev Mol Cell Biol 2*, 731–737.

Sherr C and McCormick F (2002) The RB and p53 pathways in cancer. *Cancer Cell* 2, 103-112.

Siddiqui N and Borden K (2011) mRNA export and cancer. *Wiley Interdiscip Rev RNA*, Epub ahead of print.

Steinman R and Banchereau J (2007) Taking dendritic cells into medicine. *Nature* 449, 419-426.

Stratton M, Campbell P and Futreal P (2009) The cancer genome. *Nature* 458, 719-724.

Sullivan M and Morgan D (2007) Finishing mitosis, one step at a time. *Nat Rev Mol Cell Biol* 8, 894-903.

Sun J, Yan Y, Wang X, Liu X, Peng D, Wang M et al. (2009) PTD4-apoptin protein therapy inhibits tumor growth in vivo. *International Journal of Cancer* 124, 2973-2981.

Talmadge J and Fidler I (2010) AACR centennial series: the biology of cancer metastasis: historical perspective. *Cancer Res* 70, 5649-5669.

Tavassoli M, Guelen L, Luxon B and Gaken J (2005) Apoptin: specific killer of tumor cells? *Apoptosis* 10, 717–724.

Teng M, Swann J, Koebel C, Schreiber R and Smyth M (2008) Immune-mediated dormancy: an equilibrium with cancer. *J Leukoc Biol* 84, 988-993.

Thon L, Möhlig H, Mathieu S, Lange A, Bulanova E, Winoto-Morbach S et al. (2005) Ceramide mediates caspase-independent programmed cell death. *FASEB J*, 19, 1945-1956.

Ubezio P and Civoli F (1994) Flow cytometric detection of hydrogen peroxide production induced by doxorubicin in cancer cells. *Free Radic Biol Med* 16, 509–516.

van der Eb M, Pietersen A, Speetjens F, Kuppen P, van de Velde C, Noteborn M et al. (2002) Gene therapy with apoptin induces regression of xenografted human hepatom. *Cancer Gene Ther* 9, 53-61.

van der Heiden M, Cantley L and Thompson C (2009) Understanding the Warburg effect: the metabolic requirements of cell proliferation. *Science* 324, 1029-1033.

Vilgelm A, El-Rifai W and Zaika A (2008) Therapeutic prospects for p73 and p63: rising from the shadow of p53. *Drug Resist Updat* 11, 152-163.

Vogelstein B, Lane D and Levine A (2000). Surfing the p53 network. *Nature* 408, 307-310.

Wagstaff K and Jans D (2009). Nuclear drug delivery to target tumour cells. *Eur J Pharmacol* 625, 174-180.

Wang C and Klionsky D (2003) The molecular mechanism of autophagy. *Mol Med* 9, 65-76.

Wang Z, Ahmad A, Li Y, Kong D, Azmi A, Banerjee S et al. (2010) Emerging roles of PDGF-D signaling pathway in tumor development and progression. *Biochim Biophys Acta* 1806, 122-130.

Warburg O (1925) Ueber den Stoffwechsel der Carcinomzelle. *Klin Wochenschr* 4, 534-536.

Warburg O (1956) On respiratory impairment in cancer cells. *Science* 124, 269–270.

Weinberg F and Chandel N (2009) Mitochondrial metabolism and cancer Hypoxya and consequences. *Ann NY Acad Sci* 1177, 66-73.

White E and DiPaola R (2009) The double-edged sword of autophagy modulation in cancer. *Clin Cancer Res* 15, 5308-5316.

WHO (World Health Organization) (2008) *World Cancer Report.* (P. Boyle, and B. Levin, Eds.) International Agency for Research on Cancer (IARC).

Wlodkowic D, Skommer J and Darzynkiewicz Z (2010) Cytometry in cell necrobiology revisited. Recent advances and new vistas. *Cytometry A* 77, 591-606.

Wuarin J and Nurse P (1996) Regulating S phase: CDKs, licensing and proteolysis. *Cell* 85, 785-787.

Yang L, Pang Y and Moses H (2010) TGF-β and immune cells: an important regulatory axis in the tumor microenvironment and progression. *Trends in Immunology* 31, 220–227.

Yap T, Sandhu S, Carden C and de Bono J (2011) Poly(ADP-ribose) polymerase (PARP) inhibitors: Exploiting a synthetic lethal strategy in the clinic. *CA Cancer J Clin* 61, 31-49.

Yen K, Bittinger M, Su S and Fantin V (2010) Cancer-associated IDH mutations: biomarker and therapeutic opportunities. *Oncogene* 29, 6409-6417.

Young A, Narita M, Ferreira M, Kirschner K, Sadaie M, Darot J et al. (2009). Autophagy mediates the mitotic senescence transition. *Genes Dev* 23, 798-803.

Yu J, Boyapati A and Rundell K (2001) Critical role for SV40 small-t antigen in human cell transformation. *Virology* 290, 192-198.

Yu L, Wan F, Dutta S, Welsh S, Liu Z, Freundt E et al. (2006) Autophagic programmed cell death by selective catalase degradation. *Proc Natl Acad Sci U S A*, 103, 4952–4957.

Yuan T and Cantley L (2008) PI3K pathway alterations in cancer: variations on a theme. *Oncogene* 27, 5497-5510.

Zhang Y, Kooistra K, Pietersen A, Rohn J and Noteborn M (2004) Activation of the tumor-specific death effector apoptin and its kinase by an N-terminal determinant of simian virus 40 large T antigen. *J Virol* 78, 9965-9976.

Zhang Y, Olijslagers S, Backendorf C and Noteborn MHM (2007) Apoptin close-ups: perspectives towards novel therapies. *J Med Mol Biol* 3, 401–406.

Zhao Y, Sfeir A, Zou Y, Buseman C, Chow T, Shay J et al. (2009) Telomere extension occurs at most chromosome ends and is uncoupled from fill-in in human cancer cells. *Cell* 138, 463-475.

Zhuang S, Shvarts A, van Ormondt H, Jochemsen A, van der Eb A and Noteborn M (1995) Apoptin, a protein derived from chicken anemia virus, induces p53-independent apoptosis in human osteosarcoma cells. *Cancer Research* 55, 486-48

Chapter 3

Family at last: highlights of the first international meeting on proteins killing tumour cells

P Bruno[1], CR Brinkmann[2], M-C Boulanger[3], M Flinterman[1], P Klanrit[1], M-C Landry[3], D Portsmouth[4], J Borst[5], M Tavassoli[1], M Noteborn[6], C Backendorf[6] and RME Zimmerman[6]

[1]Head and Neck Oncology Group King's College London Dental Institute, London, UK
[2]Institute of Molecular Biology, Aarhus University, Aarhus, Denmark
[3]Cancer Research Centre, University of Laval, Quebec, Canada
[4]Research Institute for Virology and Biomedicine, University of Veterinary Medicine, Vienna, Austria
[5]Netherlands Cancer Institute, Amsterdam, The Netherlands
[6]Faculty of Science, Department of Molecular Genetics, University of Leiden, Leiden, The Netherlands

Correspondence: C Backendorf, Faculty of Science, Molecular Genetics, University of Leiden, Leiden 2333CC, The Netherlands. Tel: +31 7 152 744 09; Fax: +317 152 743 40; E-mail: backendo@chem.leidenuniv.nl

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Abstract

Recently, a select number of viral and cellular proteins sharing a unique property have been identified, namely apoptin, HAMLET, TRAIL, MDA7, E4ORF4, NS1 and Brevinin-2R, where all have the remarkable ability to selectively kill transformed and/or tumour cells, while causing no or only minor cytotoxicity in normal and non-transformed cells. Research worldwide is now starting to unravel the molecular signalling pathways by which each of these proteins exerts its tumour-selective function, and the first (pre)clinical studies are very promising. The workshop 'Proteins Killing Tumour Cells' brought together, for the first time, researchers working on this novel class of intriguing proteins. Participants from 14 different countries shared their views on (possible common) mechanisms behind tumour-specific apoptosis induction and strategies to implement this knowledge in the clinic.

Cellular Transformation and Apoptosis

The ability of certain viral and cellular proteins to induce tumour-selective apoptosis implies the existence of a common set of characteristics, shared by all tumour cells, and recognized by these tumour-killing proteins (see Table 3-1). Mathieu Noteborn highlighted the delicate balance between survival and death pathways in normal and transformed cells, thereby effectively setting the theme of the meeting. Michael Green (University of Massachusetts, USA), using a genome-wide RNA interference screen, implicated secreted IGFBP7 (insulin-like growth factor binding protein 7) in the induction of senescence and apoptosis by oncogenic Braf. Paradoxically, overexpressing both proteins in normal melanocytes induced senescence, whereas in melanoma cells this resulted in apoptosis. James Pipas (University of Pittsburgh, USA) discussed the role of different domains of the SV40 large T antigen in oncogenic transformation. For instance, the N-terminal J-domain, in combination with the LXCXE domain, induces cellular proliferation by inactivating Rb and liberating E2Fs from repression. Additionally, microarray analysis revealed a new 'detoxification' function for the C-terminal domain of the large T antigen via the p450 pathway. Jason Arroyo from the Hahn group (Dana Farber Cancer Institute, USA) explained how the SV40 small T antigen contributes to the induction of transformation by inhibiting the protein phosphatase 2A (PP2A) family. Specific subunits of these heterotrimeric proteins are implicated in cell transformation: RNAi knockdown of the B56γ-subunit and mutation of the Aβ-subunit both promote transformation, whereas deletion of the Aα-subunit results in apoptosis. A common target of both transforming and tumour-selective apoptosis-inducing viral proteins appears to be the anaphase-promoting complex (APC). As illustrated by Jose Teodoro (McGill University, Canada), knockdown of the APC1 subunit by siRNA resulted in G2M cell cycle arrest and apoptosis in human tumour cells. Strikingly, this effect is mimicked by APC inactivation through interaction with either apoptin

or adenovirus E4orF4, two proteins discussed during this meeting, thus revealing a common pathway.

Proteins Killing Tumour Cells

HAMLET

Catharina Svanborg (Lund University, Sweden) introduced HAMLET (Human α-lactalbumin made lethal to tumour cells), a complex between the milk protein α-lactalbumin and oleic acid. HAMLET kills tumour and immature cells but not healthy differentiated cells by a mechanism independent of caspase activation, Bcl-2 and p53. Svanborg showed that macroautophagy is involved in HAMLET-induced cell death, as siRNA downregulation of the Beclin1 and Atg5 markers rescued HAMLET-treated cells. In clinical trials with bladder cancer patients, HAMLET exerted a direct and selective cytotoxic effect on the cancer tissue, whereas in patients with skin papillomas it resulted in complete resolution of 29 out of 35 treated papillomas. Christel Rothe Brinkmann discussed a bovine variant of HAMLET with similar cytotoxicity as the human homologue.

Brevinin-2R

Brevinin-2R-triggered cell death also involves autophagy and does not require caspase activation. The 25-aa peptide, presented by Marek Los (University of Manitoba, Canada), is a non-haemolytic defensin isolated from the skin of the frog *Rana ridibunda* and induces cell death in several cancer cell lines, with little toxicity towards primary cells.

Apoptin

Los also reported on the chicken anaemia virus-encoded protein apoptin, which induces apoptosis in numerous cancer and transformed cells, but leaves normal healthy cells unharmed. Los identified the nuclear orphan receptor Nur77 as a crucial component

of apoptin-induced cell death. In addition, he found that apoptin interacts with and activates Akt kinase, leading to nuclear localization of Akt.

Apoptin's tumour-selective cytotoxicity appears to be associated with nuclear localization and phosphorylation on T108. The nuclear targeting signal within apoptin comprises a bipartite nuclear import signal flanking a nuclear export signal. David Jans (Monash University, Australia) proposed a model where, selectively in cancer cells, NES function is inhibited by phosphorylation of apoptin on Thr108, resulting in nuclear accumulation. Dongjun Peng (Huazhong University, China) showed, in collaboration with the Leiden group, that transient expression in normal fibroblasts of the SV40 large/small T antigens is sufficient to induce apoptin's nuclear localization, phosphorylation and apoptosis. Interestingly, suppression of PP2A activity by the small T antigen (see Arroyo, above) appears to play a major role in this process. Paola Bruno from the Tavassoli group pointed out that low levels of apoptin phosphorylation can be detected in normal early passage fibroblasts infected with adenovirus expressing apoptin fused to GFP. Apparently, apoptin kinase activity is present in normal cells but has very low activity, whereas in tumour cells it is highly activated.

Rhyenne Zimmerman introduced a number of apoptin-interacting partners (AIPs) identified by the yeast two-hybrid technique. Strikingly, these AIPs are all known or putative tumour-suppressor genes or oncogenes, likely to be implicated in the differential behaviour of apoptin in normal and cancer cells. Poramaporn Klanrit showed for the first time (in collaboration with the Melino group (University of Rome, Italy) that apoptin is able to activate TAp73 and its downstream pro-apoptotic target PUMA, independently of p53 function. Joseph Cheng from the Norris group (University of South Carolina, USA), reported on the implication of the ceramide pathway

in the tumour-selective activity of apoptin. In prostate cancer cells, apoptin expression results in ceramide upregulation and acid ceramidase inhibitors can significantly enhance apoptin cytotoxicity both in prostate cancer cell lines and tumour xenografts.

Several groups discussed the use of a protein transduction strategy to deliver apoptin via the TAT protein transduction domain from HIV-1. Joop Gäken (King's College London, UK) investigated the potential of transduced apoptin to purge haemopoietic stem cells of malignant leukaemia cells, for use in autologous transplantation. His-tagged TAT-apoptin protein was shown to induce apoptosis in leukaemic cells but not in normal blood lymphocytes. Marcella Flinterman presented a novel mammalian secretable delivery system, using a modified TAT-tag. Jun Sun (Huazhong University, China) reported topical application of PTD4-apoptin onto the tumour bearing skin of immune-deficient mice. Apoptosis and disruption of the tumour integrity were apparent, whereas the surrounding normal tissue was not affected.

E4orf4

Similar to HAMLET and apoptin, over-expression of the adenoviral E4orf4 protein triggers p53-independent cell death in transformed and cancer cells, without necessitating caspase activation or mitochondrial dysfunction. However, as explained by Josée Lavoie (Laval University, Canada), E4orf4 killing requires Src tyrosine kinases and RhoGTPase-dependent perturbations of actin dynamics on endosomes. The traffic of recycling endosomes to the *trans*-Golgi is deregulated in a Cdc42- and Rab11a-dependent way, contributing to cell death. Furthermore, E4orf4 inhibits, by direct binding, v-Src-induced morphological transformation. These data support a key function for Src tyrosine kinases and actin-regulated endosome traffic in the tumour-selective killing activity of E4orf4.

TRAIL

TRAIL (tumour necrosis factor-related apoptosis-inducing ligand) selectively induces death receptor-mediated apoptosis in several cancer cell lines. Robbert Cool (University of Groningen, the Netherlands) discussed the binding of TRAIL to the death receptors DR4 and DR5, the decoy receptors DcR1, DcR2 and osteoprotegerin. To avoid sequestering by the decoy receptors, Cool developed DR5 receptor-selective TRAIL variants with improved thermal stability. Henning Walczak (DKFZ Heidelberg, Germany) introduced the TRAIL receptor agonists currently enrolled in phase I and II clinical trials, including TRAIL/Apo2L and antibodies against DR4 and DR5. TRAIL monotherapy is rarely effective, as most primary tumours seem to be TRAIL resistant. Walczak discussed how this might be circumvented by combination therapy with either chemotherapy or ionizing radiation (IR). Jannie Borst showed how TRAIL, in a soluble isoleucine-zippered form, as produced by Walczak and coworkers, enhances indeed the efficacy of radiotherapy. The apoptotic pathway induced by IR is blocked by Bcl-2, indicating that a mitochondrial contribution is required for caspase activation and cell death. Interestingly, pretreatment of Bcl-2 overexpressing cancer cells with IR makes the cells more susceptible to TRAIL-induced cell death.

MDA7

Rajagopal Ramesh (University of Texas, USA) showed that expression of the melanoma differentiation associated gene-7 (MDA7), also known as interleukin-24 (IL-24), in lung cancer and melanoma is inversely correlated with cancer progression and patient survival. MDA7/IL-24 selectively killed human cancer cells both *in vitro* and *in vivo*, with minimal effect on normal cells. In clinical phase I trials MDA7 was well tolerated and showed evidence of significant clinical activity (melanoma and solid tumours).

Parvovirus MVM-NS1

Jean Rommelaere (DKFZ, Heidelberg, Germany) discussed the tumour-specific cell killing properties of parvovirus minute virus of mice (MVM) and its encoded protein NS1, responsible for MVM-induced cytotoxicity. MVM can kill tumour cells resistant to standard cytotoxic agents such as cisplatin or TRAIL. The efficacy of MVM in a rat glioma model was illustrated by the complete remission of intracranial tumours, without side effects, and with no tumour relapse for over 1 year. Investigations of the molecular mechanism of NS1-dependent cell killing revealed that acidic vesicles accumulate in the cytosol of MVM-infected cells, leading to the cytosolic accumulation of cathepsins and activation of cathepsin B, which, in turn, leads to the activation of autophagy. NS1 exerts its toxicity by interacting with casein kinase II-α (the catalytic domain of CKII).

Conclusions

Apoptin, E4ORF4, NS1, HAMLET, TRAIL, MDA7 and Brevinin-2R are part of a new family of proteins, consisting of both cellular and viral proteins that use various mechanisms of cell death, but share one key feature: their killing activity is efficient in transformed and cancer cells and negligible in normal and healthy cells. A potential new member of this tumour killing protein family, IGFBP7, was introduced during the meeting. Besides the obvious applications for tumour targeting, these proteins also constitute very sensitive probes to zoom-in onto essential molecular and cellular changes during carcinogenic transformation. The workshop was unanimously assessed as very stimulating, bringing together for the first time various research groups working on different proteins but all with similar potentials for cancer therapeutics. As one participant stated: 'We all thought we were orphans, but now we know we are family'.

Table 3-1 Direct comparison of various properties of proteins killing tumour cells.

	Apoptin	**HAMLET**	**TRAIL**	**MDA7**	**E4orf4**	**NS1**	**Brevinin-2R**
Protein origin	Chicken anaemia virus	Complex between human milk protein - lactalbumin and oleic acid	TNF family-related apoptosis-inducing ligand	IL-10 cytokine family member	Adenovirus type 2	Parvovirus minute virus of mice (MVM)	*Rana ridibunda*
Subcellular localization	Cytoplasm (normal cells) nucleus (cancer)	Cytoplasm (normal cells) nucleus (cancer)	Extracellular receptor binding	Extracellular receptor binding	Nucleus (early) cytoplasm (late)	Cytoplasm	Cytoplasm (lysosomes)
Mechanism of (tumour-selective) cell death	Apoptosis – intrinsic pathway	Apoptosis, autophagy	Apoptosis – extrinsic pathway	Apoptosis – extrinsic pathway	Apoptosis-like	Autophagy	Autophagy
p53-dependence	No	No	No	No	No	?	?
Therapeutic status	Preclinical studies	Phase I and II clinical trials	Phase I and II clinical trials	Phase I and II clinical trials	Preclinical studies	Preclinical studies	Preclinical studies

Addendum

Since the first PKTC meeting, a number of other proteins exhibiting tumor-selective toxicity have been identified. These will be discussed below.

Noxa

Noxa is a BH3-only protein with proapoptotic activity that functions downstream of the p53-mediated apoptotic pathway. It was recently found to selectively induce apoptosis in tumor cells (Suzuki, et al., 2009). Infection with a recombinant adenovirus, contrived to express the Noxa gene, induced apoptosis in several human breast cancer cell lines *in vitro*, but not in normal mammary epithelial cell lines. Furthermore, intratumoral injection of the Noxa-expressing adenovirus resulted in marked shrinkage of transplanted tumors derived from breast cancer cells, without any notable adverse effect on the surrounding normal tissue. In contrast, the expression of Puma, another BH3-only protein that also functions downstream of the p53 pathway, induced apoptosis in both cancer and normal cells. Thus, the results suggest a mechanism wherein Noxa, but not Puma, selectively induces apoptosis in human tumor cells.

ORCTL3

The organic-cation transporter like-3 gene (ORCTL3) was identified in a systematic, high-throughput screen for genes specifically inducing cell death in transformed tumor cells (Irshad, et al., 2009). ORCTL3 was found to be inactive in normal rat kidney cells (NRK), but induced apoptosis in NRK cells transformed by oncogenic H-ras. ORCTL3 also induced cell death in v-src-transformed cells and in various human tumor cell lines but not in normal cells or untransformed cell lines. Accordingly, ORCTL3 was shown to be down-regulated in human kidney tumors. Though ORCTL3 is a member of the organic-cation transporter gene family, data indicate that it induces apoptosis independently of its putative transporter activity. Rather, experimental evidence suggest that ORCTL3-induced apoptosis is executed via an ER stress mediated mechanism.

Par-4

The prostate apoptosis response-4 (par-4) gene was first identified as an immediate early apoptotic gene, which was up-regulated in response to elevated intracellular Ca2+ concentration in rat prostate cancer cells treated with ionomycin. Human Par-4, which was found to share significant sequence similarity with its rat counterpart, was subsequently identified in yeast-two hybrid studies as a partner of the atypical Protein Kinase C (Diaz-Meco, et al., 1996) and tumor suppressor Wilm's tumor-1 (Johnstone, et al., 1996). Studies conducted in cell culture models show that over-expression of Par-4 is sufficient to directly induce apoptosis in many cancer cell types, and that this ability is associated with Par-4 nuclear translocation. Moreover, the apoptotic action of Par-4 can overcome cell protective mechanisms, such as the presence of Bcl-xL, Bcl-2, or absence of wild-type p53 or PTEN function (Shrestha-Bhattarai and Rangnekar, 2010). Interestingly, Par-4 is retained in the cytoplasm of normal and immortalized cells through an as yet unidentified mechanism, rendering it incapable of inducing apoptosis in these cells.

Accordingly, Par-4 was found to be down-regulated in many cancers, including renal cell carcinoma (Cook, et al., 1999), neuroblastoma (Kogel, et al., 2001), endometrial cancer (Moreno-Bueno, et al., 2007), and breast cancer (Zapata-Benavides, et al., 2009). Animal studies have demonstrated that Par-4 knockout mice are prone to spontaneous development of tumors in various tissues, e.g. lungs, liver, urinary bladder and endometrium, and Par-4 knockout mice are more susceptible to chemical- or hormone-induced lesions, exhibiting a significantly shorter life span compared to wild-type animals, due to death by spontaneous tumors (Garcia-Cao, et al., 2005). Par-4 is down-regulated by oncogenic Ras via the MEK-ERK pathway, and this is considered an important step towards Ras-induced transformation. Restoration of Par-4 levels, either by MEK inhibition or by stable expression of ectopic Par-4, abrogates cellular transformation. This tumor-suppressor action of Par-4 appears to be distinct from its apoptotic function (Barradas, et al., 1999; Pruitt, et al., 2005; Qiu, et al., 1999). In the case of prostate

cancer, Par-4 is not down-regulated, silenced or mutated, but inactivated due to phosphorylation by Akt1, which prevents nuclear translocation of Par-4, thereby retaining it in the cytoplasm and rendering it incapable of causing apoptosis (Goswami, et al., 2005).

In contrast, phosphorylation of the threonine 155 residue of Par-4 by Protein Kinase A (PKA), as well as its nuclear translocation, is essential for Par-4 apoptotic activity (Shrestha-Bhattarai and Rangnekar, 2010). The apoptotic effect of Par-4 involves either an activation of the cellular apoptotic machinery or inhibition of the cellular pro-survival mechanisms. Par-4 induces apoptosis in hormone-independent cancer cells by activation of the extrinsic apoptosis cascade through FADD. Therefore, in these cancer cells, over-expression of Par-4 is a sufficient signal for cell death (Chakraborty, et al., 2001). In parallel, Par-4 translocates to the nucleus and inhibits NF-κB-mediated cell survival mechanisms (Nalca, et al., 1999).

While most studies on Par-4 focused on the apoptotic effect mediated by intracellular Par-4, recent findings indicate it is also secreted by both normal and transformed mammalian cells (Burikhanov, et al., 2009). *In vitro*, Par-4 secretion could be induced by exogenous stimuli capable of causing stress in the endoplasmic reticulum (ER), and secreted Par-4 was shown to induce apoptosis via the cell-surface protein GRP78, which is coincidentally also found in the ER lumen. Par-4 was also found to be secreted *in vivo*, and could be detected in the serum of Par-4 transgenic mice. In addition, Par-4 activity in the serum was able to induce apoptosis specifically in cancer cells.

In view of these findings, both systemic (extracellular) and intracellular Par-4 may be applied for cancer therapy. In fact, TRAIL has been shown to cause the nuclear translocation of Par-4, and this mechanism is purportedly responsible for the ionizing radiation-induced bystander effect triggered in response to high-dose X-rays (Shareef, et al., 2007).

References

Barradas M, Monjas A, Diaz-Meco M, Serrano M and Moscat J (1999) The downregulation of the proapoptotic protein Par-4 is critical for Ras-induced survival and tumor progression. *EMBO J* 18, 6362–6369.

Burikhanov R, Zhao Y, Goswami A, Qiu S, Schwarze S and Rangnekar V (2009) The tumor suppressor Par-4 activates an extrinsic pathway for apoptosis. *Cell* 138, 377–388.

Chakraborty M, Qiu S, Vasudevan K and Rangnekar V (2001) Par-4 drives trafficking and activation of Fas and Fasl to induce prostate cancer cell apoptosis and tumor regression. *Cancer Res* 61, 7255–7263.

Cook J, Krishnan S, Ananth S, Sells S, Shi Y, Walther M et al. (1999) Decreased expression of the proapoptotic protein Par-4 in renal cell carcinoma. *Oncogene* 18, 1205–1208.

Diaz-Meco M, Municio M, Frutos S, Sanchez P, Lozano J, Sanz L et al. (1996) The product of par-4, a gene induced during apoptosis, interacts selectively with the atypical isoforms of protein kinase C. *Cell* 86, 777–786.

Garcia-Cao I, Duran A, Collado M, Carrascosa M, Martin-Caballero J, Flores J et al. (2005) Tumoursuppression activity of the proapoptotic regulator Par4. *EMBO Rep* 6, 577–583.

Goswami A, Burikhanov R, de Thonel A, Fujita N, Goswami M, Zhao Y et al. (2005) Binding and phosphorylation of par-4 by akt is essential for cancer cell survival. *Mol Cell* 20, 33–44.

Irshad S, Mahul-Mellier A, Kassouf N, Lemarie A and Grimm S. (2009) Isolation of ORCTL3 in a novel genetic screen for tumor-specific apoptosis inducers. *Cell Death Differ* 16, 890-898.

Johnstone R, See R, Sells S, Wang J, Muthukkumar S, Englert C et al. (1996) A novel repressor, par-4, modulates transcription and growth

suppression functions of the Wilms' tumor suppressor WT1. *Mol Cell Biol* 16, 6945–6956.

Kogel D, Reimertz C, Mech P, Poppe M, Fruhwald M, Engemann H et al. (2001) Dlk/ZIP kinase-induced apoptosis in human medulloblastoma cells: requirement of the mitochondrial apoptosis pathway. *Br J Cancer* 85, 1801–1808.

Moreno-Bueno G, Fernandez-Marcos P, Collado M, Tendero M, Rodriguez-Pinilla S, Garcia-Cao I et al. (2007) Inactivation of the candidate tumor suppressor par-4 in endometrial cancer. *Cancer Res* 67, 1927–1934.

Nalca A, Qiu S, El-Guendy N, Krishnan S and Rangnekar V (1999) Oncogenic Ras sensitizes cells to apoptosis by Par-4. *J Biol Chem* 274, 29976–29983.

Pruitt K, Ulku A, Frantz K, Rojas R, Muniz-Medina V and Rangnekar V (2005) Ras-mediated loss of the pro-apoptotic response protein Par-4 is mediated by DNA hypermethylation through Rafindependent and Raf-dependent signaling cascades in epithelial cells. *J Biol Chem* 280, 23363–23370.

Qiu S, Krishnan S, el-Guendy N and Rangnekar V (1999) Negative regulation of Par-4 by oncogenic Ras is essential for cellular transformation. *Oncogene* 18, 7115–7123.

Shareef M, Cui N, Burikhanov R, Gupta S, Satishkumar S, Shajahan S et al. (2007) Role of tumor necrosis factor-alpha and TRAIL in high-dose radiation-induced bystander signaling in lung adenocarcinoma. *Cancer Res* 67, 11811–11820.

Shrestha-Bhattarai T and Rangnekar V (2010) Cancer-selective apoptotic effects of extracellular and intracellular Par-4. *Oncogene* 29, 3873-3880.

Suzuki S, Nakasato M, Shibue T, Koshima I and Taniguchi T (2009) Therapeutic potential of proapoptotic molecule Noxa in the selective elimination of tumor cells. *Cancer Sci* 100, 759-769.

Zapata-Benavides P, Mendez-Vazquez J, Gonzalez-Rocha T, Zamora-Avila D, Franco-Molina M, Garza-Garza R et al. (2009) Expression of prostate apoptosis response (par-4) is associated with progesterone receptor in breast cancer. *Arch Med Res* 40, 595–599.

Chapter 4

Cellular partners of the apoptin-interacting protein 3 FAM96B

RME Zimmerman[1], J Sun[1,2], J Tian[1,2], M de Smit[1], AA Danen-van Oorschot[1], M Rotman[1], P Snel[1], P Voskamp[1], MHM Noteborn[1] and C Backendorf[1*]

[1]Department of Molecular Genetics, Leiden Institute for Chemistry, Leiden University, Leiden, the Netherlands. [2]Department of Biochemistry and Molecular Biology, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China

***Corresponding author:** C Backendorf, Department of Molecular Genetics, Leiden Institute of Chemistry, Leiden University, Einsteinweg 55, 2333 CC Leiden, The Netherlands. Tel: +31 715274409; Fax: +31 715274430; e-mail: backendo@chem.leidenuniv.nl

Keywords: apoptin, apoptosis, cancer, apoptin-interacting partners; chromosome segregation, DNA repair

Abbreviations: apoptin, apoptosis inducing protein; AIP3, apoptin interacting protein 3; CGI, comparative gene identification; FAM96B, Family with sequence similarity 96, member B; NER, nucleotide excision repair

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Abstract

In tumor cells apoptin is phosphorylated, locates to the nucleus and induces apoptosis in contrast to normal cells. The cellular mechanisms providing this selectivity are largely unknown. Here, we describe the identification of a new interacting partner for apoptin, apoptin interacting protein 3 (AIP3), also known as FAM96B, and examine which processes are targeted by apoptin via its interaction with this protein. We show that FAM96B interacts with apoptin independently of the latter's tumor-selective phosphorylation. Interaction studies including yeast two-hybrid and co-immunoprecipitation experiments demonstrated that FAM96B associates with proteins involved in DNA transcription, replication and repair, as well as components of the actin cytoskeleton. Furthermore, ectopic over-expression of FAM96B alone in cancer cells induced apoptosis. The results indicate that, through its interaction with FAM96B, apoptin is linked to the fundamental cellular processes of DNA repair and cell division. This discovery highlights an important part of the mechanisms responsible for tumor-selective cell death induced by apoptin.

Introduction

Recent years have witnessed the development of a new area of cancer research, focusing on proteins credited with the ability to selectively kill tumor cells (Backendorf, et al., 2009). These proteins are expected to target malignant cells more specifically than the anticancer agents that are presently used (Los, et al., 2009). Also, whereas current therapies mostly result in necrosis of the cancer cells, thus entailing a considerable amount of inflammation, these proteins induce cell death through mechanisms such as apoptosis and autophagy (Bruno, et al., 2009), potentially providing substantial improvement to the treatment of patients.

The chicken-anemia-virus derived protein apoptin has opened the field for this type of tumor-selective research. Apoptin has been shown to efficiently and safely kill tumors in various preclinical models (Grimm & Noteborn, 2010). In tumor cells, apoptin becomes phosphorylated and is located in the nucleus, whereas in normal cells it is unmodified and present in the cytoplasm (Backendorf, et al., 2008). Studies on the underlying mechanisms of apoptin-induced tumor-selective cell death have revealed that apoptin interacts with proteins such as DEDAF (Danen-van Oorschot, et al., 2004) and APC/C1 (Teodoro, et al., 2004). However, the cellular mechanisms underlying the activation of apoptin, and its subsequent execution of cell death are not yet clear (Agriris, et al., 2011).

Here, we describe that apoptin interacts with apoptin-interacting protein 3 (AIP3), previously designated FAM96B. Further analysis revealed that FAM96B itself interacts with a number of cellular proteins, mostly known to be involved in either DNA repair or cytoskeletal organization, offering important insights into the mechanisms behind tumor-selective cell death induced by apoptin.

Materials & methods

Yeast two-hybrid screens

The Matchmaker yeast two-hybrid kit System 3 was purchased from Clontech (Leusden, The Netherlands), and experiments were performed according to manufacturer protocol, with a few slight modifications. Briefly, a cDNA library derived from human keratinocytes was cloned into the leucine-selectable pGAD10yeast expression vector, downstream of the gene encoding the GAL4 activation domain (AD). Full-length apoptin or FAM96B was cloned downstream of the gene encoding the GAL4 DNA-binding domain (BD) in the tryptophane-selectable plasmid pGBKC3 and used as bait in the yeast two-hybrid screen. After proper selection, resulting clones were sequenced by LGTC (Leiden, the Netherlands).

Cells, plasmids & transfections

Positive clones from the apoptin yeast two-hybrid screen were digested with appropriate restriction enzymes to generate cDNA fragments, which were subcloned into pMT2SM-myc, providing the fragments with an in-frame N-terminal myc-tag (9E10) (Gebbink, et al., 1997) The sequence encoding myc-tagged FAM96B was then cloned into the pcDNA 3.1 (+) vector to obtain pcDNA-myc-FAM96B.

Positive clones from the FAM96B yeast two-hybrid screen were digested with appropriate restriction enzymes to generate cDNA fragments, which were subcloned into the pIBA105 vector, providing the fragments with an in-frame N-terminal strep-tag (Schmidt and Skerra, 2007) (IBA GmbH, Mannheim, Germany).

The DNA sequence encoding apoptin was synthesized by Base-Clear (Leiden, the Netherlands), according to the published apoptin sequences (Noteborn, et al., 1991) and cloned into the mammalian expression vector pcDNA. The final construct was checked by sequencing and named pcDNA-apoptin. Next, we generated the pcDNA-Flag-apoptin plasmid encoding apoptin fused with

a Flag-tag at its N-terminus. Subsequently, mutations were introduced to create flag-apoptinT108E, encoding a mutant apoptin mimicking constitutive phosphorylation, and flag-apoptin5Ala106, encoding Flag-apoptin in which amino acids 106-110 were replaced by alanines, resulting in a phosphorylation-negative mutant apoptin.

The human osteosarcoma cell line Saos-2, VH10/SV40 and human foreskin fibroblasts were grown in DMEM, supplemented with 10% newborn bovine serum (NBS), and 2 mM L-glutamine.

For transfection experiments, nucleofection technology was used in conjunction with cell type specific Nucleofector solution (AMAXA Biosystems, Cologne, Germany).

Co-immunoprecipitation and Western blot analysis

For co-immunoprecipitation experiments, cells were lysed 24h after transfection. Cells were washed with cold phosphate buffered saline and harvested in ice-cold mild lysis buffer (50mM Tris (pH 7.5), 5mM EDTA, 250mM NaCl, 0.1% Triton X-100, 5 mM NaF, 1mM Na3VO4, 20mM beta-glycerolphosphate, and Protease Inhibitor Cocktail (Roche, Almere, the Netherlands), followed by incubation on ice for 30 min. The lysate supernatant was prepared by centrifugation at 13,000 × g and 4°C for 30 min, and incubated with indicated antibodies for 90 min, followed by incubation with Protein A/G Microbeads (Miltenyi Biotech, Bergisch Gladbach, Germany). Magnetic immunoprecipitation was performed according to manufacturer protocol. Samples were resolved on sodium dodecyl sulfate-polyacrylamide gel, followed by Western blotting analysis with appropriate antibodies.

Bioinformatics analysis

The STRING database was used to identify functional network connectivity (Jensen, et al., 2009).

Results

Yeast two-hybrid analysis reveals a novel apoptin interacting protein: AIP3/FAM96B

In order to gain more insight into the mechanism by which apoptin induces apoptosis in human tumor cells, we performed a yeast two-hybrid screen using full-length apoptin as bait (Danen-van Oorschot, et al., 2004). One of the interacting proteins identified in this manner was AIP3. This protein of unknown function had previously been designated FAM96B (Finn, et al.,

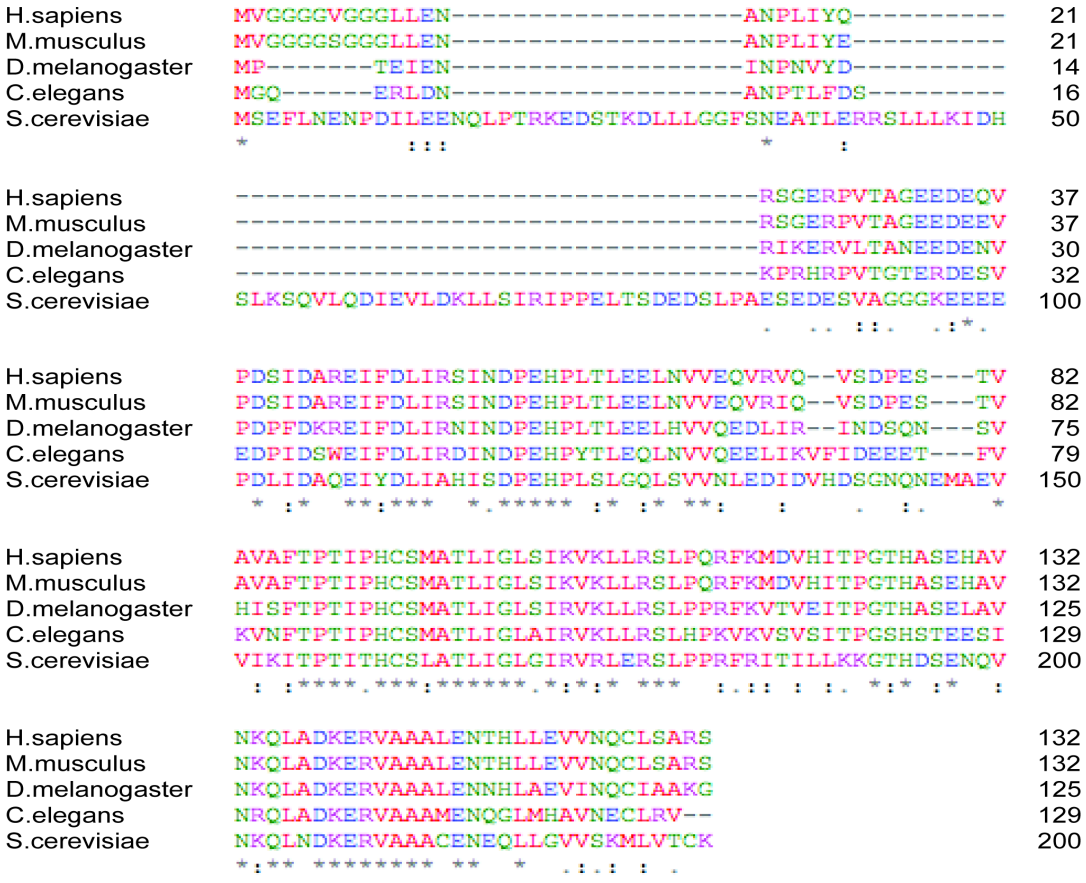

Figure 4.1 The AIP3 (also known as FAM96B) gene is conserved in chimpanzee, dog, cow, mouse, rat, chicken, zebrafish, fruit fly, mosquito, C.elegans, S.pombe, S.cerevisiae, K.lactis, E.gossypii, M.grisea, N.crassa, A.thaliana, and rice. Shown here is a ClustalW alignment of the amino acid sequences of human FAM96B (NP_057146.1) and its orthologs in the mouse Mus musculus (NP_081029.1), Drosophila melanogaster (NP_648416.1), Caenorhabditis elegans (NP_499777.1), and Saccharomyces cerevisiae (NP_011990.1). Small, hydrophobic amino acids are coloured red; acidic amino acids are blue, basic amino acids are magenta, and polar amino acids are shown in green. "*" means that the residues or nucleotides in that column are identical in all sequences in the alignment. ":" means that conserved substitutions have been observed, according to the colours indicated above. "." means that semi-conserved substitutions are observed (Chenna, et al. 2003).

2010). Figure 4.1 shows the alignment of the amino acid sequence of human FAM96B with that of the mouse, *C.elegans*, *Drosophila*, and *S.cerevisiae*, demonstrating the conservation of this protein across a wide range of species.

Apoptin interacts with FAM96B in normal and cancer cells, independently of its phosphorylation status

Next, we examined whether FAM96B associates with phosphorylated and/or non-phosphorylated apoptin in Saos-2 tumor cells and in primary fibroblasts. The cells were co-transfected with plasmids encoding Flag-tagged apoptin, Flag-apoptinT108E mimicking constitutively phosphorylated apoptin, phosphorylation deficient-mutant Flag-apoptin5Ala106 and myc-tagged FAM96B or an empty vector. Co-immunoprecipitation and Western blot analysis were carried out as described in the Materials and Methods section.

Figure 4.2A shows the Western blots of the co-immunoprecipitation analysis of normal and tumor cells expressing Flag-apoptin protein with or without FAM96B. Immunoprecipitation with antibodies against the Flag-tag of apoptin resulted for both cell types in co-immunoprecipitated FAM96B protein product. Similar co-immunoprecipitation results were obtained with lysates derived from normal and tumor cells co-expressing FAM96B and Flag-apoptinT108E or Flag-apoptin5Ala106 mutants (Figures 4.2B and C, respectively). In all cases, myc-tagged FAM96B co-immunoprecipitated with Flag-apoptinT108E or Flag-apoptin5Ala106.

These results indicate that the interaction between FAM96B and apoptin is independent of the phosphorylation status of apoptin, and can occur both in normal and cancer cells.

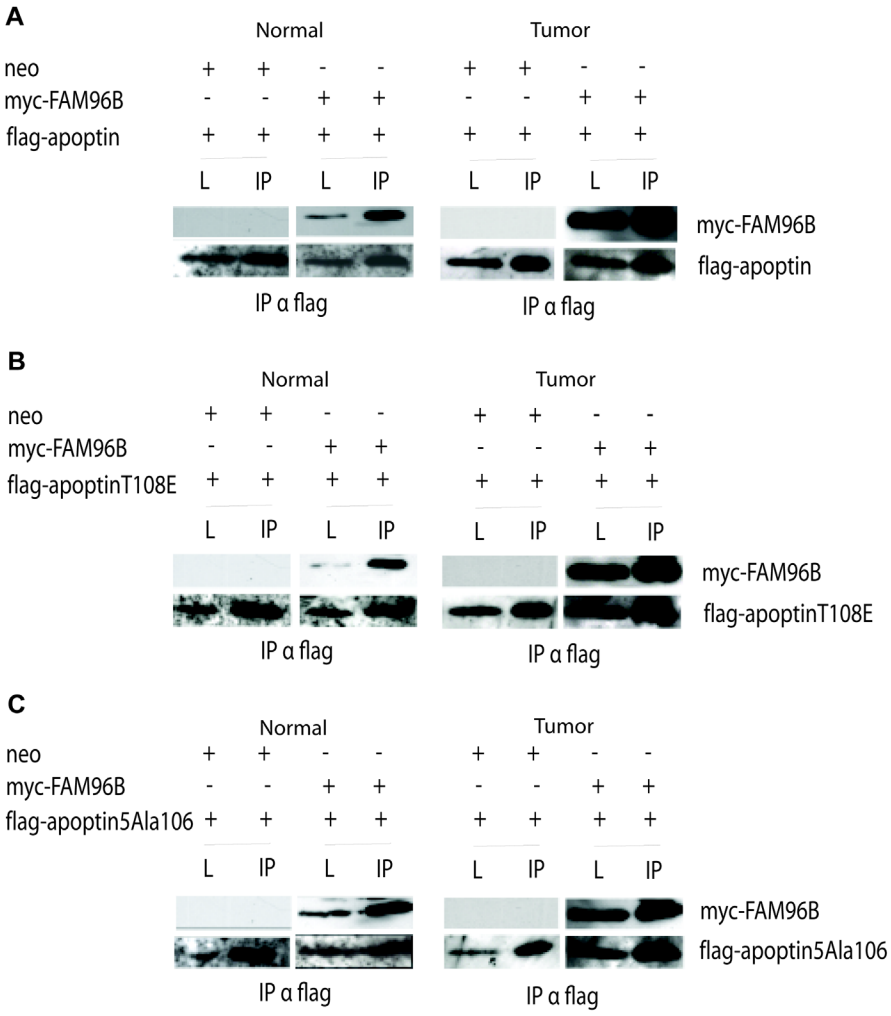

Figure 4.2 FAM96B interacts with apoptin in human cells, independently of apoptin's phosphorylation status. **A.** Cells were transfected with plasmids encoding flag-apoptin and myc-tagged FAM96B, or control plasmid (neo) in the indicated combinations. Total lysates (L) or protein complexes immunoprecipitated with antibody against the flag-tag were separated by SDS-PAGE and analyzed by Western blotting. *Left panel* normal human foreskin fibroblasts; *right panel* human Saos-2 tumor cells. **B.** As **A**, using flag-apoptinT108E, which mimicks constitutively phosphorylated apoptin. **C.** As **A,** using flag-apoptin5Ala106, in which amino acids 106-110 have been replaced with a 5-aa stretch of alanines, thereby abolishing the phosphorylation site at aa108.

Association of FAM96B with DNA repair and the cytoskeleton

A yeast two-hybrid screen was then performed using human FAM96B as bait, with the purpose of understanding the cellular processes involving FAM96B, and how these could be involved in the induction of apoptosis by apoptin. Twenty different proteins were found to associate with FAM96B (Table 4-1). For all but one of these interactants, the association could be confirmed by co-immunoprecipitation in a human cellular background (Figure 4.3).

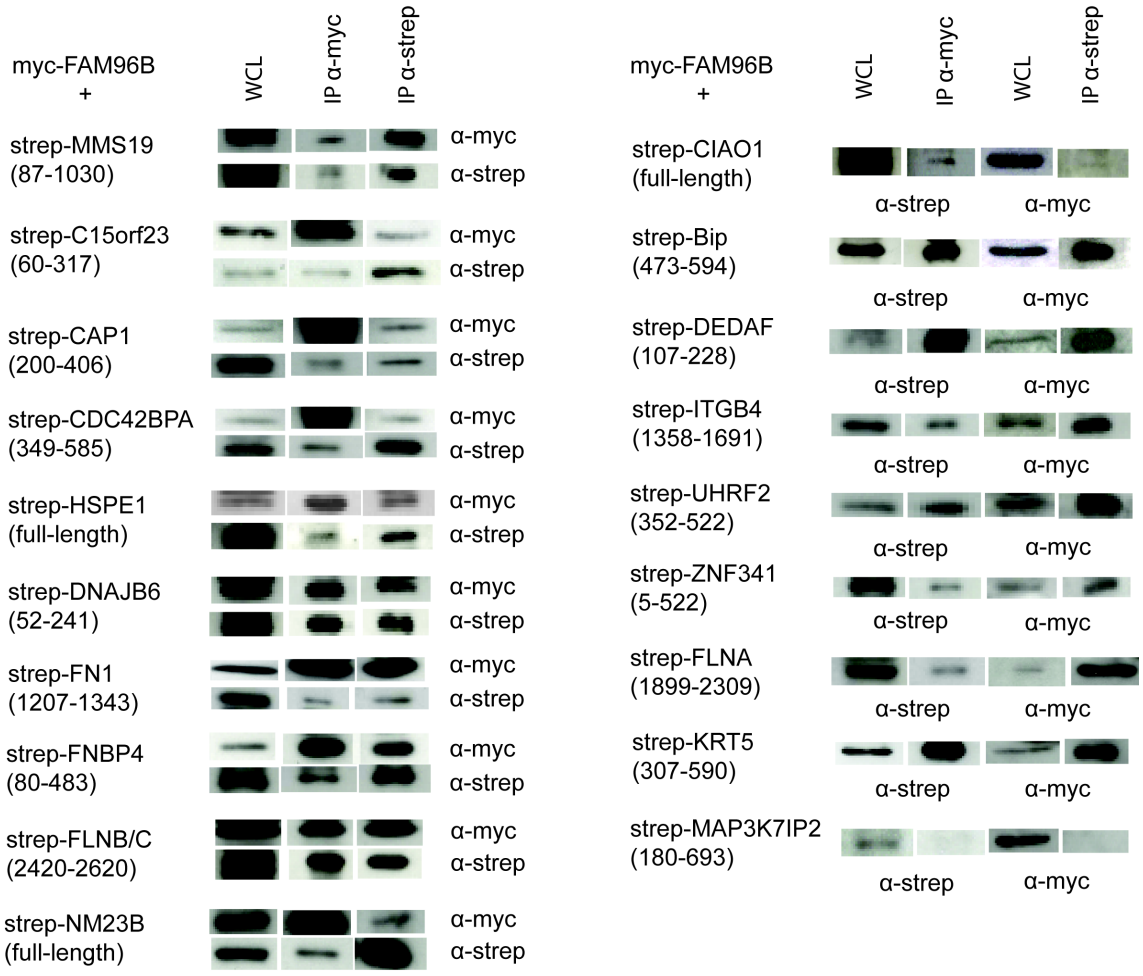

Figure 4.3 Co-immunoprecipitation of FAM96B in human cells with interaction partners identified by yeast two-hybrid. VH10/SV40 cells were transfected with plasmids encoding myc-tagged FAM96B and strep-tagged candidate interaction partners. Twenty-four hours post transfection, cells were lysed and protein complexes immunoprecipitated with antibodies recognizing either the myc- or strep-tag. MAP3K7IP2 was the only candidate partner out of 20 that did not interact with FAM96B in a human cell background.

Bioinformatics analysis of the cellular pathways in which the newly identified FAM96B interactants were involved, revealed the distribution of these partners in two main groups of proteins, namely DNA transcription and repair, and cytoskeleton organization and dynamics, with many linked specifically to chromosome segregation (Table 4-2 and Figure 4.4). Among these proteins, there are clear links to tumor-related processes: the nucleoside diphosphate kinase NM23B, for instance, has been shown to suppress metastasis (Roymans, et al., 2002), while UHRF2 is a putative tumor suppressor gene (Bronner, et al., 2007; Sjöblom, et al., 2006), and CIAO1 is linked to the Wilms tumor suppressor protein 1 (WT1) (Johnstone,

et al,1998). FAM96B was also found to associate with protein chaperones such as BIP (HSPA5) and HSPE1 (Hsp10), both of which have been shown to be differentially expressed in cancer (Zhuang, et al., 2009; Czarnecka, et al., 2006), Interestingly, both BIP and DEDAF, a transcriptional repressor involved in DNA damage-induced apoptosis, has previously also been shown to interact with apoptin, and over-expression of DEDAF in tumor cells has been shown to induce apoptosis (Danen- van Oorschot, et al., 2004; Novak and Phillips, 2008; Sato, et al., 2010).

As depicted in figure 4.4, there are also many links between the FAM96B interactants themselves. From this network of proteins, it emerges that FAM96B might be involved in the regulation of cell division, particularly chromosome segregation, and that this regulation is likely subject to DNA repair pathways.

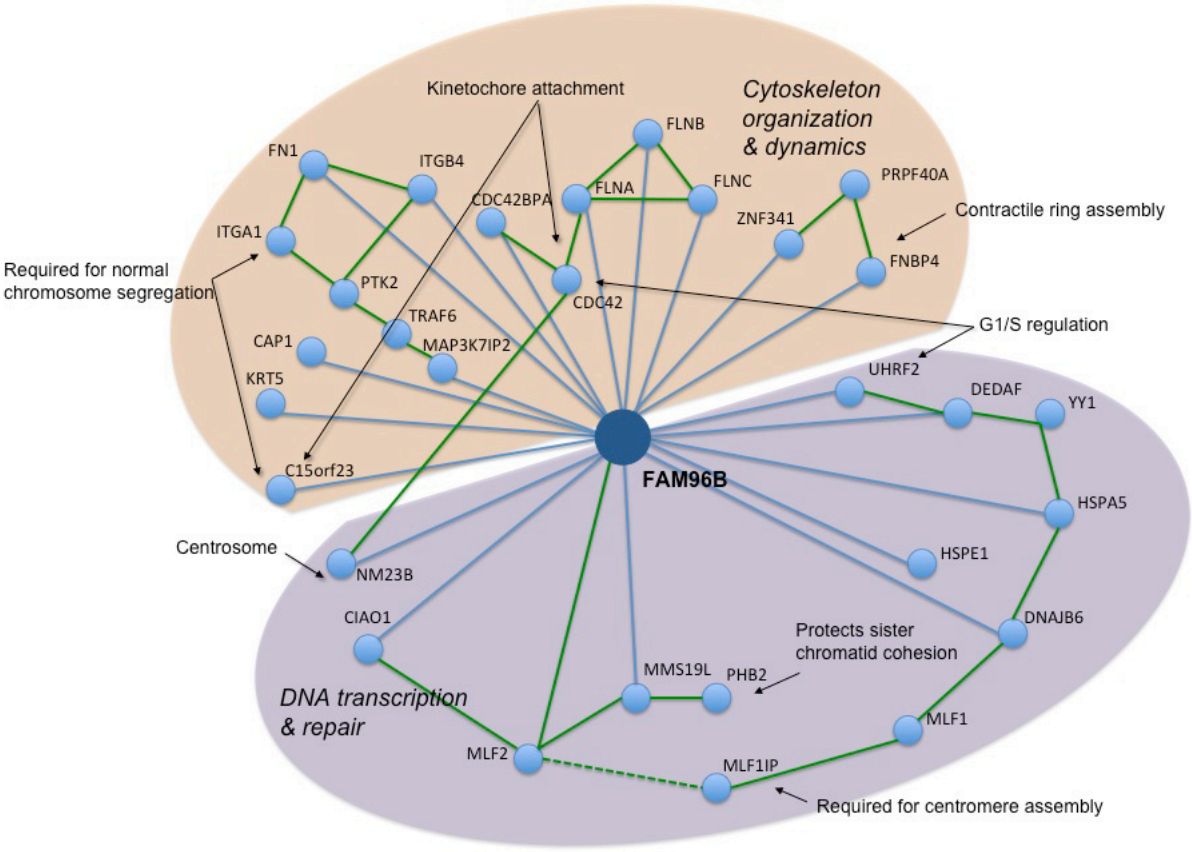

Figure 4.4 Schematic representation of the FAM96B interaction network, as derived from our yeast two-hybrid experiments (blue lines) and literature (green lines). The STRING database was used to identify functional network connectivity (Jensen, et al. 2009).

Table 4-1 Proteins associating with FAM96B. FAM96B interactants were identified through yeast two-hybrid analysis and confirmed as FAM96B partners in a human cellular background. Besides the protein name, the table also lists the proposed function (Funcbase annotation according to Beaver, et al., 2010) and, if applicable, links to cancer.

Protein name	Proposed function	FuncBase annotation	Links to cancer
CIAO1	Cytosolic Fe–S cluster maturation, transcriptional regulation: Depletion of Cia1 impairs the export of the large ribosomal subunit from the nucleus	Positive regulation of cell proliferation	Deletion lethal in S. cerevisiae. Modulates the transactivation activity of Wilms tumor suppressor protein (WT1)
NM23B	Nucleoside diphosphate kinase	Metastasis suppressor	Suppressor of metastasis
MMS19	Nucleotide excision repair and POLII transcription	Nucleotide excision repair	Common variation associated with increased risk of pancreatic cancer. May also function as transcriptional co-activator of estrogen receptor (ER)
DEDAF (RYBP)	PcG protein with transcriptional co-repressor activity Induces cell-cycle arrest and is involved in the p53 response to DNA damage	Transcriptional repressor	Decreased in human cancer tissues
UHRF2 (NIRF)	E3 ubiquitin protein ligase Induces G1 arrest and associates with Cdk2/cyclin E	E3 ubiquitin ligase, cell cycle regulator	Putative tumor suppressor gene
MAP3K7IP2 (TAB2)	MAPK signaling pathway (Links MAP3K7 with TRAF6; also interacts with TRAF2	NF kappa B signaling	
ZNF341	May be involved in transcriptional regulation		
DNAJB6 (MRJ)	DNA J-domain containing molecular chaperone	Regulation of caspase activity, unfolded protein reponse, intermediate filament cytoskeletal reorganization	Expression is lost in breast cancer Over-expression partially reverses mesenchymal phenotype and reduces malignant activity of breast cancer
CAP1	Directly regulates filament dynamics	Cell morphology and motility	Over-expression is involved in aggressive behavior of pancreatic cancer
CDC42BPA	Serine/threonine protein kinase	Actin cytoskeleton reorganization	May act as a downstream effector of CDC42 in cytoskeletal reorganization, contributes to cell invasion
FN1	Cell adhesion and motility	Cell adhesion and migration	
FNBP4	Formin binding protein	Cell cycle checkpoint	

Table 4-1. continued.

ITGB4	Laminin receptor, structural role in the hemi-desmosomes of epithelial cells	Cytokine binding, cell migration	Likely to play a pivotal role in the biology of invasive carcinoma.
FLNA/B/C	Connects cell membrane constituents to the actin cytoskeleton	Actin cytoskeleton	Aberrant FLN's are involved in e.g. acute myelomonoblastic leukaemia
KRT5	Cytokeratin	Cytoskeletal keratin; cell migration	
BIP	Heat shock protein; probably plays a role in the assembly of multimeric protein complexes in the ER	Unfolded protein response	Possibly involved in pathogenesis of renal cell carcinoma, expression appears to correlate with melanoma progression
HSPE1	Mitochondrial heat shock protein	Unfolded protein response, caspase regulation	Expression up-regulated in cancer
C15orf23 (SKAP)	Associates with kinetochores; promotes metaphase-to-anaphase transition		

Table 4-2 FAM96B-associating proteins are grouped according their biological function. Protein names in *italics* are assigned to more than one group, and the asterisk (*) denotes the only protein identified by yeast two-hybrid, for which the interaction could not be confirmed through co-immunoprecipitation in a human cellular background.

DNA transcription & repair	**Cytoskeleton organization & dynamics**
CIAO1 *NM23B* MMS19 DEDAF UHRF2 MAP3K7IP2* ZNF341	*CIAO1* *NM23B* *DNAJB6* C15orf23 CAP1 CDC42BPA FN1 FNBP4 ITGB4 FLNA FLNB FLNC KRT5
Protein chaperone	
BIP HSPE1 *DNAJB6*	

FAM96B induces apoptosis in cancer cells

To investigate this last hypothesis, we analyzed the effect of FAM96B over-expression on cancer cells. As the DNA repair pathways in cancer cells are presumed to be faulty, we expected over-expression of FAM96B in these cells to halt cellular proliferation. To this end, human cancer cells were transfected with plasmids encoding myc-tagged FAM96B and green fluorescent protein (GFP) as a negative control. At twenty-four, forty-eight and seventy-two hours after transfection, cells were fixed and stained with DAPI to determine apoptosis activity. Twenty four hours after transfection, a small percentage of FAM96B-positive cells exhibited morphological features charactristic of apoptosis, steadily increasing up to approximately 40% after 72 hours. In contrast, GFP-expressing cells did not exceed the level of 5% morphologically apoptotic cells. The results clearly demonstrate that expression of FAM96B induced apoptosis in cancer cells, whereas the negative control GFP did not (Figure 4.5).

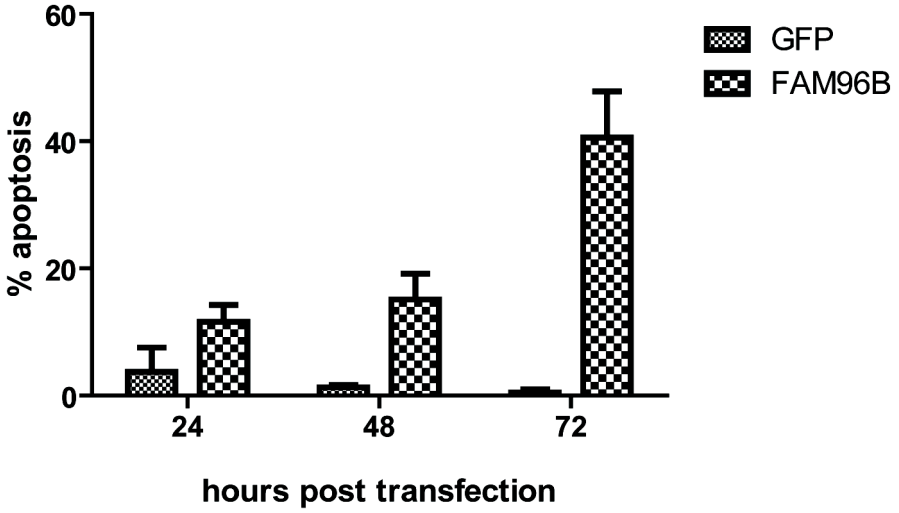

Figure 4.5 Over-expression of FAM96B in apoptosis in human cancer cells. Human Saos-2 tumor cells were transfected with plasmid encoding myc-tagged FAM96B or control and grown on coverslips. Twenty-four, forty-eight, and seventy-two hours post transfection coverslips were fixed and stained with appropriate antibodies for immunofluorescence analysis. Apoptotic cells were scored according to their nuclear morphology as determined by DAPI staining. Data is representative of 3 independent experiments, each in which at least 100 cells were scored.

Discussion

We have identified cellular factors providing insights in the action of the tumor-selective apoptosis inducer apoptin. Our protein-protein interaction studies revealed firstly the association of apoptin with human FAM96B, and secondly of FAM96B with proteins involved in DNA transcription, replication and repair as well as cytoskeleton dynamics. The fact that FAM96B interacts with apoptin in both its phosphorylated and non-phosphorylated state indicates its importance to apoptin's activity: the interaction likely occurs in an already early phase, preceding apoptin's phosphorylation and activation of its tumor-selective apoptosis induction.

Though the exact function of FAM96B cannot yet be established, several clues are provided. FAM96B is highly conserved among eukaryotes, and contains a well conserved domain (DUF59), found in proteins serving various functions, including phenylacetic acid degradation, iron-sulfur cluster biosynthesis, and calcium-dependent protein kinase, but also nucleotide-binding and chromosome partitioning (Finn, et al., 2010). Studies in *S.cerevisiae* suggest that the yeast homolog of FAM96B is an essential protein (Davierwala, et al., 2005), and further analysis even indicates its requirement for the establishment of sister chromatid cohesion (Ben-Aroya, et al., 2008). Recently, Ito and colleagues have reported that a protein complex containing FAM96B is involved in chromosome segregation (Ito, et al., 2010).

Our analyses concur with these findings, indicating a role for FAM96B in linking chromatin-related processes to the cytoskeleton. For example, one of the proteins identified as an FAM96B interactor is filamin A, a cytoskeletal protein, which has recently been shown to interact with the tumor suppressor protein BRCA1 and is required for efficient DNA repair (Velkova, et al., 2010). We found that FAM96B associates with MMS19, which itself is required for nucleotide excision repair (NER) and RNA polymerase II transcription (Kou, et al., 2008). Through MMS19 and DNAJB6, FAM96B is

also linked to PHB2 and MLF1IP, respectively, which have functions in protecting chromatid cohesion (Takata, et al., 2007) and centromere assembly (Foltz, et al., 2006). Another FAM96B-protein interactor, NM23B, has been identified as a constituent of the centrosome (Roymans, et al., 2001), and connects both FAM96B interaction spheres on account of its roles in transcription as well as regulation of cytoskeleton dynamics and suppression of metastasis (Lim, et al., 1998). Importantly, FAM96B-interactor C15orf23, or SKAP, has been shown to promote metaphase-to-anaphase transition by mediating separase activation after spindle assembly checkpoint is satisfied in mitosis (Fang, et al., 2009).

These findings are further supported by the fact that FAM96B is linked to formin, which is essential for contractile ring assembly in animal cells (Glotzer, 2005), as well as Cdc42, which regulates the assembly and organization of the actin cytoskeleton, and is required for kinetochore attachment as well as G1 progression and S phase entry (Hall, 2009). UHRF2, with which FAM96B interacts directly, is also capable of inducing G1 arrest, and associates with CDK2 (Li, et al., 2004). CDK2 complexed with cyclins A and E is essential for DNA replication and G1/S transition, respectively (Sherr, 1994). Intriguingly, Maddika, et al. (2009) reported apoptin phosphorylation and activation by CDK2.

We therefore propose that FAM96B functions in the regulation of cellular proliferation through control of chromosome segregation, and that this regulation is subject to control by DNA repair pathways. Consistent with this proposed function, we also showed that over-expression of FAM96B in cancer cells induces apoptosis. This apoptotic activity is highest 72 hours post transfection, and thus most likely represents a late-phase response, presumably upon failure to activate and/or execute a proper DNA damage response in a tumorigenic environment.

In conclusion, our protein-protein interaction analysis revealed that apoptin interacts with FAM96B, which appears to link the cell division process to the

essential process of DNA repair. Further research is underway, seeking to characterize and more precisely define the role of FAM96B in these processes, and the involvement in apoptin-induced tumor-selective cell death.

Note added in proof:
During preparation of this manuscript, Ito et al. described that FAM96B (named MIP18 by the authors) is part of a multiprotein complex involved in DNA repair and chromosome segregation in human cells (Ito, et al., 2010).

Acknowledgements

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References

Agriris K, Panethymitaki C and Tavassoli M (2011) Naturally occurring, tumor-specific, therapeutic proteins. *Exp Biol Med* 236, 524-536.

Backendorf C, Noteborn MHM, Tavassoli M. Proteins killing tumour cells. (2009) Transworld Research Network, Kerala, India.

Backendorf C, Visser A, De Boer A, Zimmerman R, Visser M, Voskamp P et al. (2008) Apoptin: Therapeutic potential of an early sensor of carcinogenic transformation. *Annu Rev Pharmacol Toxicol* 48, 143-169.

Beaver J, Tasan M, Gibbons F, Tian W, Hughes T, and Roth F. (2010) FuncBase: A resource for quantitative gene function annotation. *Bioinformatics* 26, 1806-1807.

Ben-Aroya S, Coombes C, Kwok T, O'Donnell K, Boeke J, and Hieter P (2008) Toward a comprehensive temperature-sensitive mutant repository of the essential genes of Saccharomyces cerevisiae. *Mol Cell* 30, 248-258.

Bronner C, Achour M, Arima Y, Chataigneau T, Saya H, Schini-Kerth V et al. (2007) The UHRF family: oncogenes that are drugable targets for cancer therapy in the near future? *Pharmacol Ther* 115, 419–434.

Bruno P, Brinkman CR, Boulanger MC, Flinterman M, Klanrit P, Landry MC et al. (2009) Family at last: highlights of the first international meeting on proteins killing tumour cells. *Cell Death Diff* 16, 184-186.

Chenna R, Sugawara H, Koike T, Lopez R, Gibson TJ, Higgins DG et al. (2003) Multiple sequence alignment with the Clustal series of programs. *Nucleic Acids Res* 31, 3497-3500.

Czarnecka AM, Campanella C, Zummo G, Cappello F. (2006) Heat shock protein 10 and signal transduction: a "capsula eburnea" of carcinogenesis? *Cell Stress Chaperones* 11, 287-294.

Danen-van Oorschot A, Voskamp P, Seelen M, van Miltenburg M, Bolk M, Tait S et al. (2004) Human death effector domain associated factor interacts with the viral apoptosis agonist Apoptin and exerts tumor-preferential cell killing. *Cell Death Differ* 11, 564-573.

Davierwala A, Haynes J, Li Z, Brost R, Robinson M, Yu L et al. (2005) The synthetic genetic interaction spectrum of essential genes. *Nat Genet* 37, 1147-1152.

Fang L, Seki A, Fang G. (2009) SKAP associates with kinetochores and promotes the metaphase-to-anaphase transition. *Cell Cycle* 8, 2819-2827.

Finn R, Mistry J, Tate J, Coggill P, Heger A, Pollington J et al. (2010) The Pfam protein families database. *Nucleic Acids Res* 38, D211-222.

Foltz D, Jansen L, Black B, Bailey A, Yates J 3 and Cleveland D (2006) The human CENP-A centromeric nucleosome-associated complex. *Nat Cell Biol* 8, 458-469.

Gebbink M, Kranenburg O, Poland M, van Horck F, Houssa B, Moolenaar W (1997) Identification of a novel, putative Rho-specific GDP/GTP exchange factor and a RhoA-binding protein: control of neuronal morphology. *J Cell Biol* 137, 1603-1613.

Glotzer, M. (2005) The molecular requirements for cytokinesis. *Science* 307, 1735-1739.

Grimm S, Noteborn M (2010) Anticancer genes: inducers of tumour-specific cell death signalling. *Trends Mol Med* 16, 88-96.

Hall A (2009) The cytoskeleton and cancer. *Cancer Metastasis Rev* 28, 5-14.

Ito S, Tan LJ, Andoh D, Narita T, Deki M, Hirano Y et al. (2010) MMXD, a TFIIH-independent XPD-MMS19 protein complex involved in chromosome segregation. *Cell* 39, 632-640.

Jensen L, Kuhn M, Stark M, Chaffron S, Creevey C, Muller J, et al. (2009) STRING 8--a global view on proteins and their functional interactions in 630 organisms. *Nucleic Acids Res* 37, D412-416.

Johnstone R, Wang J, Tommerup N, Vissing H, Roberts T, and Shi Y (1998) Ciao 1 is a novel WD40 protein that interacts with the tumor suppressor protein WT1. *Biol Chem* 273, 10880-10887.

Kou H, Zhou Y, Gorospe R, and Wang Z (2008) Mms19 protein functions in nucleotide excision repair by sustaining an adequate cellular concentration of the TFIIH component Rad3. *Proc Natl Acad Sci U S A* 105, 15714-15749.

Li Y, Mori T, Hata H, Homma Y and Kochi H (2004) NIRF induces G1 arrest and associates with Cdk2. *Biochem Biophys Res Commun* 25, 464-468.

Lim S, Lee H, and Lee H (1998) Inhibition of colonization and cell-matrix adhesion after nm23-H1 transfection of human prostate carcinoma cells. *Cancer Lett* 133, 143-149.

Los M, Panigrahi S, Rashedi I, Mandal S, Stetefeld J, Essmann F, et al. (2009) Apoptin, a tumor-selective killer. *Biochim Biophys Acta* 1793, 1335-1342.

Losada A, Hirano M and Hirano T (1998) Identification of Xenopus SMC protein complexes required for sister chromatid cohesion. *Genes Dev* 12, 1986-1997.

Maddika S, Panigrahi S, Wiechec E, Wesselborg S, Fischer U, Schulze-Osthoff K, et al. (2009) Unscheduled Akt-triggered activation of cyclin-dependent kinase 2 as a key effector mechanism of apoptin's anticancer toxicity. *Mol Cell Biol* 29, 1235-1248.

Noteborn M, de Boer G, van Roozelaar D, Karreman C, Kranenburg O, Vos J et al. (1991) Characterization of cloned chicken anemia virus DNA that contains all elements for the infectious replication cycle. *J Virol* 65, 3131-3139.

Novak R, and Phillips A (2008) Adenoviral-mediated Rybp expression promotes tumor cell-specific apoptosis. *Cancer Gene Ther* 15, 713-722.

Roymans D, Vissenberg K, De Jonghe C, Willems R, Engler, G, Kimura N et al. (2001) Identification of the tumor metastasis suppressor Nm23-H1/Nm23-R1 as a constituent of the centrosome. *Exp Cell Res* 262, 145-153.

Roymans D, Willems R, Van Blockstaele D, and Slegers H (2002) Nucleoside diphosphate kinase (NDPK/NM23) and the waltz with multiple partners: possible consequences in tumor metastasis. *Clin Exp Metastasis* 19, 465-476.

Sato M, Yao V, Arap W, and Pasqualini R (2010) GRP78 signaling hub a receptor for targeted tumor therapy. *Adv Genet* 69, 97-114.

Schmidt T and Skerra A (2007) The Strep-tag system for one-strep purification and high-affinity detection or capturing of proteins. *Nat Protoc* 2, 1528-1535.

Sherr C (1994) G1 phase progression: cycling on cue. *Cell* 79, 551–555.

Sjöblom T, et al. (2006) The consensus coding sequences of human breast and colorectal cancers. *Science* 314, 268-274.

Takata H, Matsunaga S, Morimoto A, Ma N, Kurihara D, Ono-Maniwa R et al. (2007) PHB2 protects sister-chromatid cohesion in mitosis. *Curr Biol* 17, 1356-1366.

Teodoro JG, Heilman DW, Parker AE and Green MR (2004) The viral protein apoptin associates with the anaphase-promoting complex to induce G2/M arrest and apoptosis in the absence of p53. *Genes Dev* 18, 1952-1957.

Velkova A, Carvalho M, Johnson J, Tavtigian S and Monteiro A (2010) Identification of filamin A as a BRCA1-interacting protein required for efficient DNA repair. *Cell Cycle* 9, 1421-1433.

Zhuang L, Scolyer R, Lee C, McCarthy S, Cooper W, Zhang X, et al. (2009) Expression of glucose-regulated stress protein GRP78 is related to progression of melanoma. *Histopathology* 54, 46

Chapter 5

Apoptin interaction with chromatin

RME Zimmerman[1], BI Florea[2], C Backendorf[1], MHM Noteborn[1,*]

[1]Department of Molecular Genetics and [2]Bio-organic Synthesis, Leiden Institute for Chemistry, Leiden University, Leiden, The Netherlands

***Corresponding author**: MHM Noteborn, Department of Molecular Genetics, Leiden Institute of Chemistry, Leiden University, Einsteinweg 55, 2333 CC Leiden, The Netherlands. Tel: +31 715274544; Fax: +31 715274430; e-mail: m.noteborn@lic.leidenuniv.nl

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Abbreviations: apoptin, apoptosis inducing protein; ATR, ataxia telangiectasia mutated and Rad3 related; CAV, chicken anemia virus; Cdc5L, cell cycle regulated phosphatase 5L; Cdk, cyclin-dependent kinase; ChIP, chromatin immunoprecipitation; Chk1, checkpoint kinase 1; DBPA, DNA-binding protein A; DDX5, dead box proteins p68; DEDAF, death effector domain-associated factor; DEDD, DED-containing DNA-binding protein; EEF/EIF, translation elongation and initiation factors; hnRNP, heterogenous nuclear ribonuclear proteins; FAM96B, Family with sequence similarity 96, member B; HCMV, human cytomegalovirus; HPV, human papilloma virus; HSV, herpes simplex virus; HTLV, Human T-lymphotropic virus**;** MDM2, mouse double minute 2 protein; MS, mass spectrometry; mTOR, mechanistic target of rapamycin, serine/threonine kinase; Nur77 orphan nuclear

receptor 77; NONO, non-POU domain containing, octamer-binding; NPM, nucleophosmin; PCBP2, poly(rC)-binding protein 2; PP2A, protein phosphatase 2a; pRb, retinoblastoma susceptibility protein; RP, ribosomal protein; RRP1B, ribosomal RNA processing 1 homolog B; SAF-A, scaffold attachment factor A; SARS, Severe Acute Respiratory Syndrome; SV40, simian virus 40; U2AF, U2 snRNP auxiliary factor

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Abstract

The Chicken Anemia Virus-derived protein apoptin has been identified as one of a select number of proteins preferentially exhibiting toxicity towards cancer cells. Coupled to this tumor-selective behavior is a differential subcellular localization: in tumor cells, apoptin-induced apoptosis involves its nuclear translocation, whereas in normal cells, it remains cytoplasmic, where it is degraded without killing the cell. To obtain a comprehensive nuclear interaction map of apoptin in the cancer cell nucleus, and shed light on the mechanisms underlying its tumor cell-killing abilities, we designed a proteomic strategy based on chromatin immunoprecipitation (ChIP) coupled with mass spectrometry. We found that apoptin localizes to chromatin containing proteins relevant for ribosome biogenesis, RNA metabolism, DNA damage response and cell cycle regulation. Our data suggest that in transformed cells, apoptin might induce apoptosis from within the nucleolus, where it senses the activation of the DNA damage response and interferes with cellular protein synthesis.

Introduction

The viral protein apoptin has emerged as a promising new instrument in the development of effective anti-cancer therapies (Backendorf, et al., 2009; Li, et al., 2010; Wagstaff and Jans, 2009). Apoptin has been shown to induce apoptosis specifically in tumor cells, compared to normal tissue (Danen-van Oorschot, et al., 1997; Sun, et al., 2009). Apoptosis induction by apoptin is preceded by its phosphorylation and nuclear accumulation (Danen-van Oorschot, et al., 2003; Maddika, et al., 2009; Rohn, et al., 2002;). *In-vitro* experiments with recombinant MBP-apoptin have demonstrated that apoptin is able to interact with both single- and double-stranded DNA, with little or no sequence specificity (Leliveld, et al., 2004). Intriguingly, however, experiments using actinomycin D, an RNA synthesis inhibitor, and two protein synthesis inhibitors (emetine and puromycin), demonstrated that apoptin's cell-killing effects did not require *de novo* transcription or translation (Danen-van Oorschot, et al., 2003). Furthermore, apoptin specifically requires a tumorigenic nucleus: enforced nuclear localization of apoptin in normal human cells by fusion of a general nuclear localization signal to apoptin (Danen-van Oorschot, et al., 2003) or by direct micro-injection of recombinant MBP-apoptin protein in the nucleus (Zhang, 2004a) did not result in apoptosis. In other experiments, apoptin remained in the cytoplasm of normal human cells, and only translocated to the nucleus upon cellular transformation by ectopic expression of the oncogenic SV40 large T antigen (Zhang, 2004b).

In view of these properties, we aimed to further characterize the nuclear activities of apoptin, and how these result in apoptosis induction in cancer cells. We present evidence that chromatin-bound apoptin complexes contain proteins involved in ribosome biogenesis and RNA metabolism, the DNA damage response pathway and regulation of the cell cycle. Taken altogether, our data indicate that

apoptin might coordinate its tumor-specific apoptosis-inducing activity from inside the nucleolus.

Materials & methods

Cell lines, plasmids and transfections

The human SV40 transformed fibroblast cell line VH10/SV40 (Klein, et al., 1990) was grown in DMEM, supplemented with 10% newborn bovine serum (NBS). The human normal diploid skin fibroblast strain F44 (up to passage 15) was grown in 1:1 DMEM/Ham's F12, supplemented with 10% fetal calf serum (FCS), and 2 mM L-glutamine. All culture media were obtained from Invitrogen (Breda, The Netherlands) and contained penicillin and streptomycin.

Plasmid pcDNA-Flag-apoptin containing the DNA sequences encoding Flag-tagged apoptin have been described elsewhere (Zimmerman, et al., 2011a). For transfection experiments, nucleofection technology was used in conjunction with cell type specific Nucleofector solution (AMAXA Biosystems, Cologne, Germany), according to manufacturer instructions.

Immunofluorescence

Cells were grown on glass cover slips. After transfection, the cover slips were first washed once with phosphate buffered saline, and subsequently fixed with methanol/acetone (50%/50%) for 10 min at room temperature. After air-drying, the slides were used for immunocytochemical staining or stored at -20°C for further analysis. Immunocytochemical staining was carried out as described by Danen-Van Oorschot, et al. (1997). Antibody α-Flag (Sigma-Aldrich, Zwijndrecht, Netherlands), a mouse monoclonal antibody against the Flag-tag fused to apoptin, was used to detect the presence and cellular localization of apoptin. The fluorescein isothiocyanate (FITC) or Rhodamine conjugated goat antibodies (Jackson ImmunoResearch

Laboratories, Suffolk, UK) were used as secondary antibodies. At least 100 cells were scored per sample in two independent experiments.

In vivo cross-linking

Twenty-four hours after transfection, SV40-transformed VH10 fibroblasts were subjected to *in vivo* cross-linking and chromatin immunoprecipitation (Fousteri, et al., 2006). All procedures were carried out at 4°C unless otherwise stated. Briefly, 3×10^7 cells were cross-linked with 1% formaldehyde (HCHO) prepared from an 11% stock (0.05 M HEPES (pH 7.8), 0.1 M NaCl, 1 mM EDTA, 0.5 mM EGTA, 11% HCHO) for 16 min. Next, 0.125 M (final concentration) of a glycine solution was added, and the cells were collected by scraping in cold PBS. All buffers used for cell extraction and ChIP contained, in addition to the specified components, 0.1 mM EDTA, 0.5 mM EGTA, 1 mM PMSF, and a mixture of proteinase and phosphatase inhibitors. The cell pellet was resuspended in lysis (CL) buffer (50 mM HEPES (pH 7.8), 0.15 M NaCl, 0.5% NP-40, 0.25% Triton X-100, and 10% glycerol) and rotated for 10 min. After centrifugation (1300 rpm, 5 min), the supernatant was removed. The pellet was washed with buffer consisting of 0.01 M Tris-HCl (pH 8.0), 0.2 M NaCl, 0.5 mM DTT, and resuspended in 1 × RIPA buffer (0.01 M Tris-HCl (pH 8.0), 0.14 M NaCl, 1% Triton X-100, 0.1% Na-deoxycholate, 0.1% SDS). The nuclear suspension was sonicated on ice using a Branson Sonifier 250 (Danburry, CT, USA), yielding fragments between 200 and 1000 bp. The supernatant containing the cross-linked chromatin was collected by centrifugation (13200 rpm, 10 min) and stored in aliquots at -80°C.

Chromatin Immunoprecipitation

For each ChIP reaction, an equal amount of cross-linked chromatin was immunoprecipitated with 0.5–2 μg of the specific anti-Flag antibody or mouse IgG (negative control) in RIPA buffer during o/n incubation. The immunocomplexes were collected by adsorption (3 hr)

to pre-cleared protein G Sepharose beads (Upstate Biotechnology, Inc., Lake Placid, NY, USA) in RIPA buffer containing 0.1 mg/ml sonicated salmon sperm DNA (ssDNA). The beads were next washed twice with 20 vol of RIPA, once with RIPA containing ssDNA, and twice with RIPA containing ssDNA and 0.3 M NaCl. Finally, the beads were washed with 20 vol of LiCl buffer (0.02 M Tris (pH 8.0), 0.25 M LiCl, 0.5% Triton X-100, 0.5% Na-deoxycholate) and the immunocomplexes were resuspended in TE buffer.

Mass Spectrometry

The proteins obtained by ChIP analysis were identified by mass spectrometry (MS) analysis as follows. The ChIP samples were processed by chloroform-methanol precipitation (Wessel and Flugge, 1984), then solubilized in urea and submitted to in-solution proteolytic digestion according to the filter-aided sample preparation (FASP) protocol using a 10K filter, as described by Wiśniewski, et al. (2009). Before proceeding with MS, samples were purified, desalted and concentrated using StageTips (Rappsilber, et al., 2007). The samples were analyzed on a LTQ-orbitrap mass spectrometer (Thermo-Fischer, Breda, the Netherlands) and MS and MSMS was run. Sequence identification was performed using Mascot Daemon (Matrix Science Inc, London, United Kingdom).

Results and Discussion

Apoptin localizes to specific regions of the cancer cell nucleus

We set out to gain a better understanding of the mechanisms behind tumor-selective apoptosis induction by apoptin, starting with its particular nuclear localization. Earlier studies have shown that, in human cancer cells, apoptin forms distinct intranuclear granules, localizing to heterochromatin and nucleoli (Leliveld, et al., 2003). In order to gain further evidence for these findings, we transfected both normal and SV40-transformed human fibroblasts with plasmid DNA

encoding flag-tagged apoptin. Twenty-four hours after transfection, cells were fixed and the subcellular localization of apoptin was determined by immunofluorescence assay. As shown in figure 5.1, apoptin was localized in the cytoplasm of the normal cells, whereas in the transformed cells, apoptin exhibited its characteristic intranuclear localization pattern.

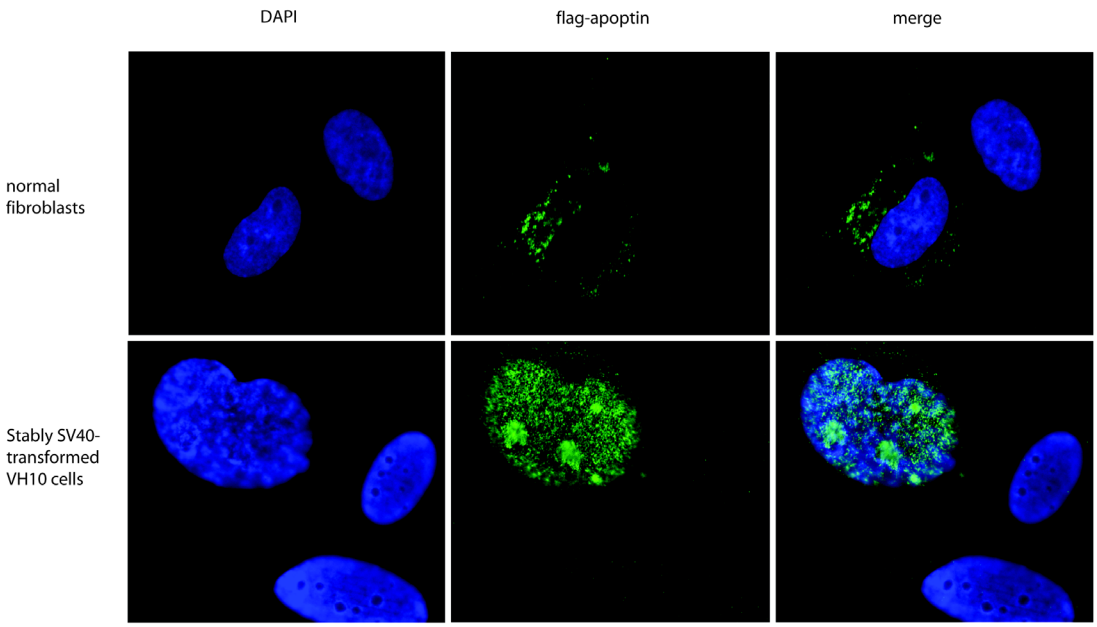

Figure 5.1 Differential localization of apoptin in normal and transformed cells. Normal fibroblasts (upper panel) or stably SV40-transformed fibroblasts (lower panel) were transfected with plasmid DNA encoding flag-tagged apoptin. Twenty-four hours post-transfection, cells were fixed and stained for indirect immune fluorescence analysis as indicated in the Materials & Methods section.

Chromatin Immunoprecipitation identifies the interaction of apoptin with various nuclear proteins

Next, we examined whether apoptin could be found in specific protein complexes interacting with nuclear chromatin. To this end, we designed a proteomic strategy based on chromatin immunoprecipitation (ChIP) coupled with mass spectrometry (Figure 5.2). SV40-immortalized fibroblasts were transfected with flag-apoptin, and chromatin-protein complexes were precipitated using an antibody against the flag-tag. As a negative control, chromatin-protein complexes were incubated with human IgG.

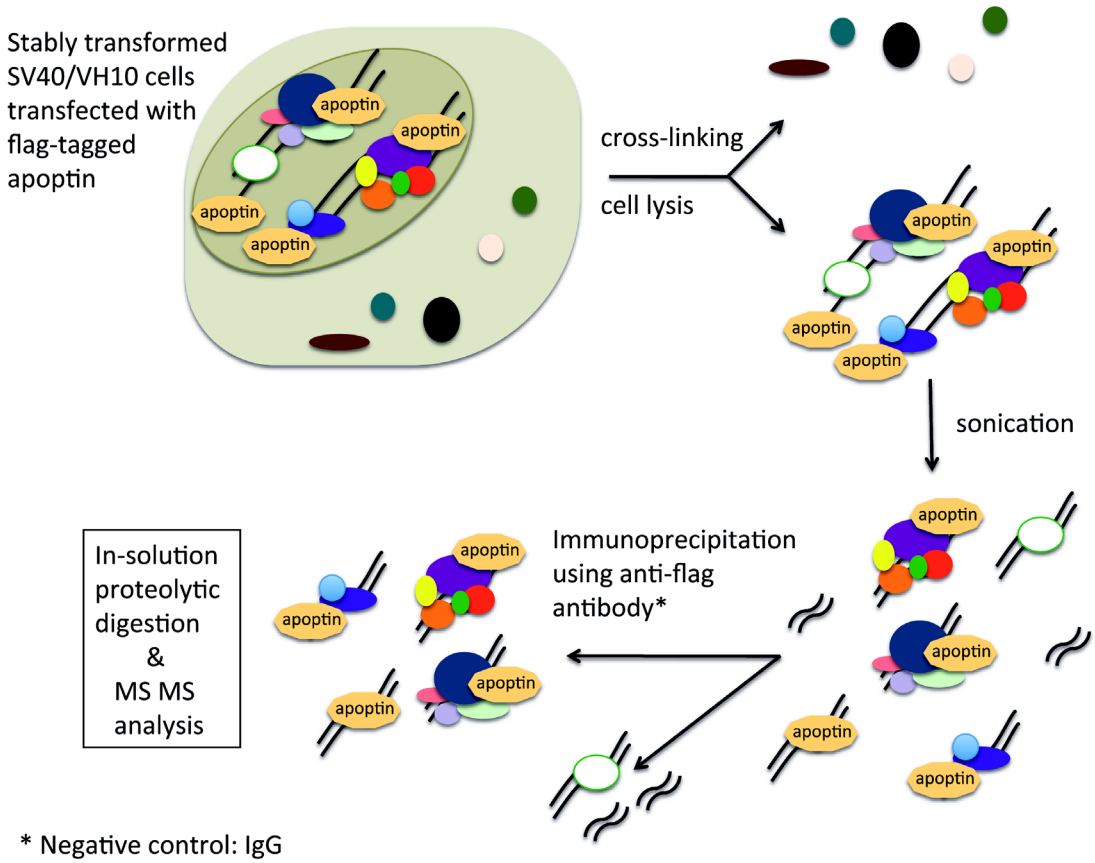

Figure 5.2 Schematic representation of the proteomics approach undertaken in our study to obtain a comprehensive nuclear interaction map of apoptin in the cancer cell nucleus. Stably SV40-transformed fibroblasts were transfected with flag-tagged apoptin. Twenty-four hours post transfection, proteins were cross-linked to chromatin using 1% formaldehyde, after which cells were lysed. Nuclei were pelleted, washed, resuspended, then sonicated to yield chromatin fragments between 200 and 1000 bp. Size of DNA fragments was checked by DNA gel electrophoresis (data not shown). Apoptin-bound complexes were precipitated using an anti-flag antibody* (recognizing the flag-tag fused to apoptin). After in-solution proteolytic digestion, proteins were identified using mass spectrometry analysis. *Alternatively, proteins were incubated with mouse IgG as a negative control.

Several proteins, which were clearly absent in the negative control, were found to co-precipitate with chromatin-associated apoptin complexes (Figure 5.3). Following in-solution digestion, the identity of these proteins was determined by mass spectrometry analysis. Proteins identified in the negative control were considered aspecific to the apoptin-chromatin interaction, and were excluded from further analysis. The list of remaining proteins, specifically present in chromatin-bound apoptin complexes, is presented in Table 5-1.

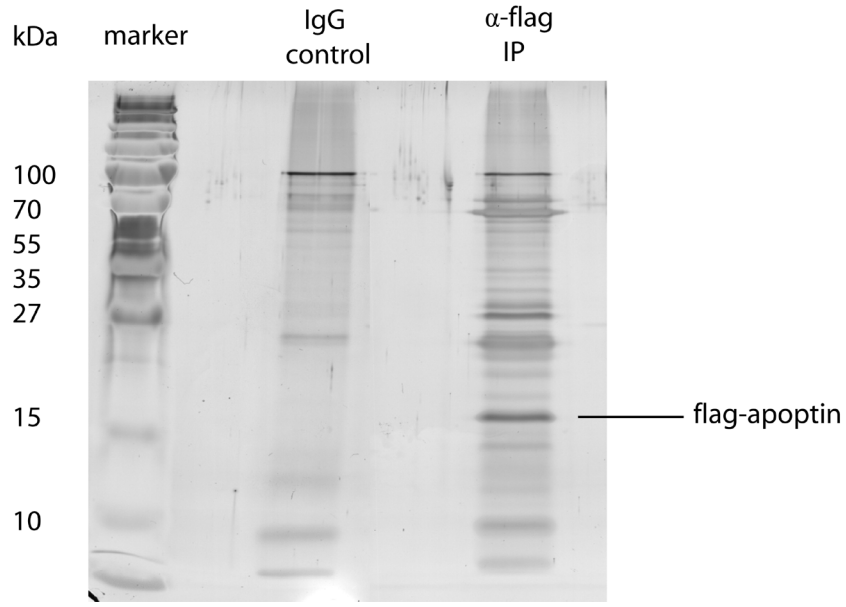

Figure 5.3 Interaction profile of chromatin-bound apoptin complexes, isolated from SV40-transformed cells, 24 hours post transfection with flag-tagged apoptin. Chromatin immunoprecipitation was carried out using flag-antibody (bait) or mouse IgG (control). Specifically interacting proteins were subsequently resolved by SDS-PAGE and silver-stained according to manufacturer protocol (Bio-Rad, Hercules, California, USA). The resulting protein profile obtained with the anti-flag antibody is specific and composed of bands of distinct size and intensity, representing putative proteins interacting with chromatin-bound apoptin. The band belonging to flag-apoptin is indicated (this was determined by western blot analysis, data not shown).

The identification of histone proteins proves that we indeed precipitated chromatin-bound apoptin complexes, while the identification of nucleolar proteins is consistent with the previously observed nucleolar localization of apoptin in cancer cells. Additionally, apoptin has previously been shown to interact with importin beta-1 (Poon, et al., 2005) and tubulin alpha and beta (Teodoro, et al. 2004), confirming the specificity of our analysis. Many of the identified proteins have been demonstrated to harbor cell cycle-related functions and possess either oncogenic or tumor suppressive activities. In addition, several of these proteins have been shown to interact with viral proteins, and are involved in the replication of viral genomes.

Table 5-1 Proteins identified by mass spectrometry analysis of purified apoptin-chromatin immunocomplexes. Proteins unspecifically coprecipitating with the IgG negative control were subtracted from this list.

	Human proteins			
Mass (kDa)	**Protein name**	**Alternative names**	**Function**	**Links to cancer**
14.1 13.9 15.4	histone H2a histone H2B histone H3.2		Nucleosome constituent	
16.5 23 14.9 26.8 30.2 22.1 29.8 15.4 18 18.6 14.8 17.9 14.5 12.6 34.5 29.2	40S ribosomal protein S16 40S ribosomal protein S5 40S ribosomal protein S15a 40S ribosomal protein S3 40S ribosomal protein S3a 40S ribosomal protein S7 40S ribsosomal protein S4, x 40S ribosomal protein S24 60S ribosomal protein L12 60S ribosomal protein L21 60S ribosomal protein L22 60S ribosomal protein L24 60S ribosomal protein L31 60S ribosomal protein L35A 60S acidic ribosomal protein P0 60S ribosomal protein L7		Ribosome constituent	

Table 5-1 continued.

Mass (kDa)	Protein name	Alternative names	Function	Links to cancer
38.9	hnRNP A1		Formation, packaging, processing, and nuclear-cytoplasmic transport of mRNA	Over-expression is associated with non-small cell lung cancer. (Boukakis, et al., 2010)
36.3	hnRNP A/B			
37.5	hnRNP A2/B1			Over-expressed in non-small cell lung cancer (Boukakis, et al., 2010), gastric cancer (Jing, et al., 2011), and hepatocellular carconoma (Cui, et al., 2010).
33.7	hnRNP C1/C2			
46	hnRNP F			Expression is up-regulated in colon cancer (Balasubramani, et al., 2006)
49.5	hnRNP H			
60.7	hnRNP L			
77.7	hnRNP M			
91.2	hnRNP U	SAF-A		
39	PCBP2	hnRNP E2		Down-regulated in oral cancer (Roychoudhury, et al., 2007)
28.4	U2AF		Splicing factor	
54.3	NONO	p54NRB	RNA-binding protein, which plays various roles in the nucleus, including transcriptional regulation and RNA splicing, as well as the DNA damage response	Highly expressed in malignant melanoma (Shiffner, et al., 2011)
16.7	NFAT5		Transcription factor	Activity promotes carcinoma invasion (Jauliac, et al., 2002)
40.1	DBPA	CSDA		Up-regulated in gastric cancer (Wang, et al., 2009)
50.8	EEF1A2		Eukaryotic translation initiation and elongation	Putative oncogenes (Lee and Surh, 2009; Lew, et al., 1992; Tang, et al., 2010).
50.4	EEF1G			
17	EIF5A-1,2			

Table 5-1 continued.

Mass (kDa)	**Protein name**	**Alternative names**	**Function**	**Links to cancer**
76.6 32.7 19.6	Nucleolin Nucleophosmin Nucleophosmin-3	C23 B23, NPM1 NPM3	Nucleolar proteins, involved in the synthesis and maturation of ribosomes, as well as regulation of many other cellular processes, including DNA damage repair, cell cycle and apoptosis. See text for further details.	
33.5	SET	TAF-I	PP2A inhibitor, involved in the regulation of cell cycle progression; see text for further details.	
92.4	CDC5L		Splicing factor and essential regulator of G2/M progression (Bernstein and Coughlin, 1998). Also regulates S-phase checkpoint in response to DNA damage (Zhang, et al., 2009).	
69.6	DDX5	p68	DEAD-box RNA helicase implicated in cellular growth and division; see text for further details.	Overexpressed in prostate cancer (Clark, et al., 2008)
122.9	A26C1		Pro-apoptotic actin isoform (Liu, et al., 2009)	Overexpressed in hepatocellular carcinoma (Chang, et al., 2006)
28.3 50	Filamin A Tubulin alpha, beta		Cytoskeletal proteins	
58.2 98.4	Importin subunit alpha-2 Importin subunit beta 1		Nuclear protein import	
	Viral proteins			
Mass (kDa)	**Protein name**			
13.5 82.4	apoptin SV40 large T antigen			

To facilitate interpretation, we grouped the proteins identified in our apoptin ChIP assay into the following 8 functional groups (Table 5-1, Figure 5.4): 1) Histones; 2) Ribosome constituents and other proteins involved in ribosomal biogenesis; 3) hnRNPs; 4) translation factors; 5) transcription factors; 6) regulatory nucleolar proteins; 7) proteins regulating progression through the cell cycle; 8) cytoskeletal proteins.

Below, we will discuss a select number of these proteins in more detail, highlighting possible novel insights into the mechanisms underlying tumor-selective cell killing by apoptin.

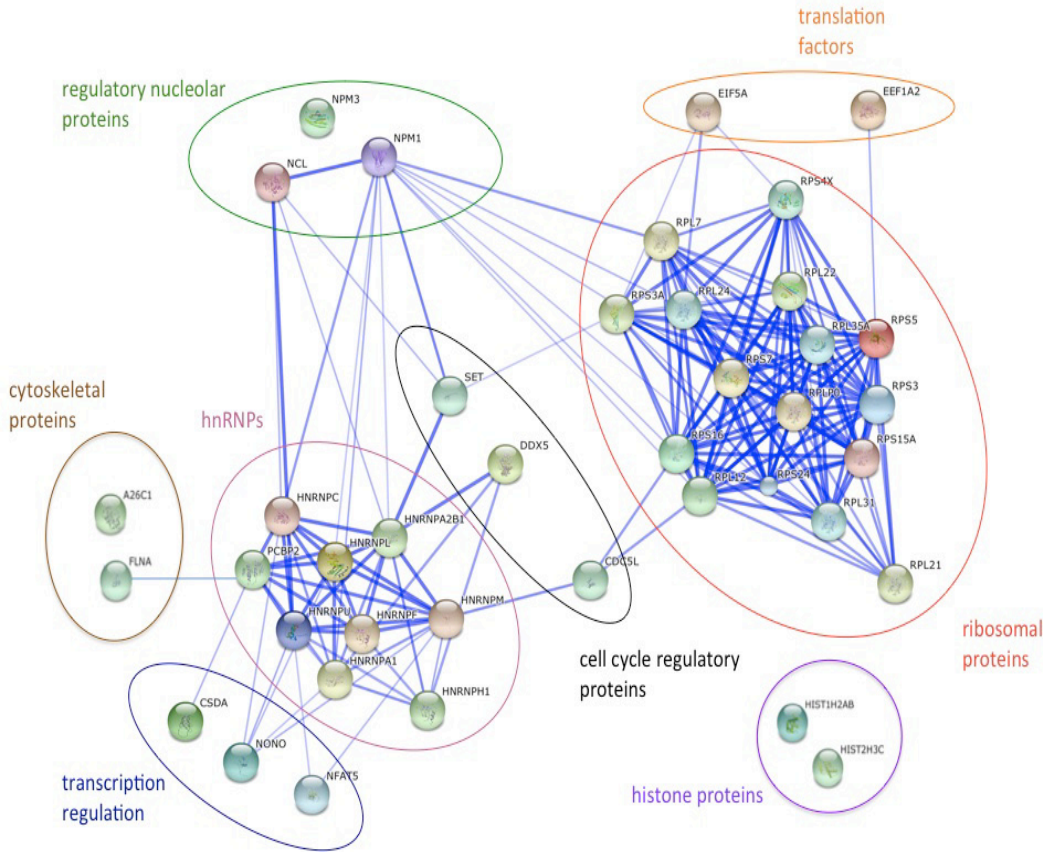

Figure 5-4 The STRING database (Jensen, et al. 2009) was used to identify functional network connectivity among the proteins identified by apoptin chromatin immunoprecipitation. See text for further details.

Apoptin: functions in and out of the nucleolus

Ribosome biogenesis

The nucleolus is generally regarded as a cellular hub for ribosomal biogenesis, vital to cellular proliferation (Montanaro, et al., 2008). The identification of apoptin in chromatin containing ribosomal constituents confirms not only its presence at this important hub, but also its propensity for a transformed environment, given that the presence of multiple, large nucleoli has long been regarded as an immunohistochemical hallmark of cancer cells (Pianese, 1896). In fact, emerging evidence suggests that quantitative and qualitative changes in rRNA synthesis may be among the most important molecular alterations occurring in cancer cells, and several approved anticancer therapeutics are proposed to exert their activity at least in part through interference with this process (Drygin, et al., 2010). For instance, cisplatin, which is traditionally believed to work primarily through the induction of DNA damage, was shown to block rRNA synthesis through inhibition of the rRNA polymerase Pol-I; in contrast, its clinically ineffective analog transplatin, although still being able to damage DNA, had no effect on rRNA synthesis (Jordan and Carmo-Fonseca, 1998). This data imply a role for apoptin interference with ribosome biogenesis as part of its mechanism for inducing apoptosis in cancer cells.

Sensing cellular stress/DNA damage

Recent research has provided evidence that, more than a mere ribosome factory, the nucleolus actively contributes to the regulation of cellular survival and proliferation, playing crucial roles in many fundamental cellular processes, including (regulation of) DNA repair, cell cycle checkpoints in mitosis, and apoptosis (Boisvert, et al., 2007; Boulon, et al., 2010). These pathways are mainly influenced through sequestration of regulators, such as p53, MDM2, and pRB (Tembe and Henderson, 2007). A group of ribosomal proteins (RPs), including

RPL5, RPL11, RPL23 and RPS7, were shown to serve as stress signal transmitters: following stress, they are released from the nucleolus and bind Mdm2, thereby activating p53 (Zhang and Lu, 2009). RPS7 was among the many ribosomal proteins identified in our apoptin chromatin precipitation assay, as was nucleophosmin, a major nucleolar phosphoprotein that has also been shown to act as a nucleolar stress sensor (Yao, et al., 2010). Nucleophosmin associates with all three components of the p14(ARF)-p53-MDM2 cascade, (Bertwistle, et al., 2004; Colombo, et al., 2005; Kurki, et al., 2004), and is further linked to the DNA damage response through its interaction with the checkpoint kinase Chk1 (Chen, et al., 2009). It is also involved in numerous other cellular processes, including ribosome biogenesis (Okuwaki, et al., 2002). Nucleophosmin-regulated ribosome export is a fundamental process in cell growth, and inhibition of nucleophosmin shuttling can block cellular proliferation (Maggi, et al., 2008). It is plausible that the interaction with apoptin achieves just this, thereby contributing to tumor-selective apoptosis induction by apoptin.

Regulation of mitosis

Our assay also identified the nucleophosmin-interacting protein nucleolin, a major nucleolar phosphoprotein with important roles in chromatin structure, rDNA transcription, rRNA maturation, nucleocytoplasmic transport, and ribosome assembly (Ginisty, et al., 1999; Liu and Yung, 1999; Srivastava and Pollard, 1999). Both nucleolin and nucleophosmin are highly expressed in proliferating and cancerous cells (Eichler and Craig, 1994; Pianta, et al., 2010).

Aside from their nucleolar functions, both proteins have important functions in the regulation of mitosis. Nucleolin localizes to the chromosome periphery in mitotic cells, is associated with the spindle poles from prometaphase to anaphase, and is involved in chromosome congression and spindle formation (Ma, et al., 2007), while

nucleophosmin is present at centromeres (Foltz, et al., 2006) and at spindle poles in metaphase cells, and is required for proper chromosome alignment on the equatorial planes during metaphase (Rousselet, 2009). Nucleophosmin is also required for centrosome duplication (Moss and Stefanovsky, 2002; Okuda, et al., 2000), the formation of functional and stable spindles with intact centrosomes and for proper kinetochore-microtubule attachments (Amin, et al., 2008). Apoptin was previously shown to interact with FAM96B, which also localizes to the mitotic spindle and is required for proper sister chromatid cohesion and chromosome segregation (Zimmerman, et al., 2011b; Ito, et al, 2010). The association with nucleolin and nucleophosmin, as well as actin, filamin and tubulin, again places apoptin at the mitotic spindle, further accentuating its important mechanistic function in apoptin-induced apoptosis.

Further evidence comes from the identification of Cdc5L and SET in our assay. Cdc5L is the human homolog of Schizosaccharomyces pombe Cdc5, and like its yeast counterpart, it is an important cell cycle regulator of G2/M transition (Bernstein and Coughlin, 1998). It is also required for progression through the S-phase of the cell cycle, with recent studies describing a function for Cdc5L in the cellular response to DNA damage (Zhang, et al., 2005), demonstrating that it interacts physically with the cell-cycle checkpoint kinase ataxia-telangiectasia and Rad3-related (ATR), and is required for the activation of downstream effectors or mediators of ATR checkpoint function, including Chk1 and Rad17 (Zhang, et al., 2009). SET, a potent inhibitor of the tumor suppressor protein phosphatase 2A (PP2A) (Li, et al., 1996) and an important modulator of chromatin remodeling and condensation, also regulates progression through the cell cycle (Vera, et al., 2007; Leung, et al., 2011). It binds directly to p21CIP1 and reverts the inhibitory effect of the latter protein on cyclin E-Cdk2 kinase activity, thus allowing progression through S-phase (Estanyol, et al., 1999). SET also regulates G2/M transition by

binding to cyclin B and inhibiting cyclin B-Cdk1 activity (Canela, et al., 2003). Hence, through its interactions with nucleophosmin, nucleolin, Cdc5L, and SET, and their roles in the pathways regulating the DNA damage response as well as progression through mitosis, we gain valuable new insights into apoptin's *modus operandi* inside the cancer cell nucle(ol)us.

hnRNPs and other RNA-related proteins (RNPs)

In our chromatin immunoprecipitation experiments apoptin was also found to associate with a large number of heterogeneous nuclear ribonucleoproteins (hnRNPs).

hnRNPs comprise a family of RNA-binding proteins that are principally involved in RNA metabolism (Han, et al., 2010). Within the nucleus, hnRNP proteins participate in RNA splicing, 3'-end processing, transcriptional regulation, and immunoglobulin gene recombination. hnRNP proteins are also involved in nucleo-cytoplasmic transport of mRNAs, mRNA localization, translation and mRNA stability. Recently, evidence has been provided that hnRNPs are also associated with heterochromatin, at least in Drosophila (Piacentini, et al., 2009). hnRNP-U has been found to associate with the heterochromatin protein 1 alpha (HP1 alpha) in both the nucleoplasm and in chromatin (Ameyar-Zazoua, et al., 2009); hnRNP-A2/B1 was shown to associate with DNA-bound proteins (Guha, et al., 2009); and hnRNP-C1/C2 proteins were demonstrated to bind to chromatin in a DNA damage-dependent manner (Wardleworth and Downs, 2005). Additionally, it has been suggested that hnRNP proteins play critical and well-defined roles in carcinogenesis. hnRNP-E1, -A1, and -D have been shown to form a heteromeric complex on telomeric repeats *in vitro* (Ishikawa, et al. 1993), suggesting that they may be involved in the maintenance of telomeric length. This notion is reinforced by the observation that deficient expression of hnRNPs can cause significant telomere shortening and oncogenic transformation

(LaBranche, et al. 1998). A recent study has indeed confirmed that hnRNP-A1 participates in telomere maintenance (Flynn, et al., 2011). hnRNP-A2/B1 is over-expressed in lung, breast, pancreas and liver cancer (Tauler, et al., 2010), and hnRNP-A1 has been identified as a potential biomarker for colorectal cancer (Ma, et al., 2009). Moreover, hnRNP-E1, hnRNP-K, and FUS (or hnRNP-P2) were identified as intricate constituents of spreading initiation centers, which are ribonucleoprotein complexes involved in the initiation of cell spreading during cancer metastasis (de Hoog, et al. 2004). Down-regulation of hnRNP-E1 stimulated cell spreading, consistent with the observation that shRNA-mediated silencing of hnRNP-E1 induces constitutive EMT, another prerequisite for metastatic progression (Chaudhury, et al. 2010; de Hoog, et al. 2004). Reduced hnRNP-E1 expression is also a prerequisite for human papillomavirus (HPV) proliferation and subsequent incidence of cervical carcinoma from cervical dysplasia (Pillai, et al. 2003). A study on the molecular mechanisms by which hnRNP proteins regulate cell proliferation in cancer, showed that hnRNP-A1 and hnRNP-A2 proteins control the alternative splicing of pyruvate kinase mRNA, which facilitates the metabolic shift from oxidative phosphorylation to aerobic glycolysis in cancer (Chen, et al., 2010). Another study demonstrated that hnRNP-F was involved in the regulation of cell proliferation via the mTOR/S6 kinase 2 pathway (Goh, et al., 2010). Finally, importantly, hnRNP-U (SAF-A), is specifically phosphoryated in response to double-stranded DNA breaks (Berglund and Clarke, 2009), and was shown to be a novel spindle regulator with an essential role in kinetochore-microtubule attachment and mitotic spindle organization (Ma, et al., 2011).

The identification of this many RNPs in our assay likely signifies an important association between apoptin and the regulation of RNA metabolism, a conclusion which is further strengthened by the above-described, recently discovered roles of hnRNPs in carcinogenesis.

Viral replication

As briefly alluded to before, several of the proteins identified in our assay have been shown to interact with viral genomes and proteins, and take part in the process of viral replication (see also Table 5-2).

Table 5-2 Proteins involved in viral replication identified by ChIP-MS analysis of chromatin-bound apoptin protein complexes.

Protein	*Relevant to viral replication of*
Nucleolin	Human papilloma virus-16 Herpes simplex virus-1 Cytomegalovirus
DDX5	SARS virus Hepatitis C virus
PCBP2	Hepatitis C virus Dengue virus
hnRNP F	Influenza A virus
hnRNP C	Poliovirus
hnRNP A1	HLTV-1

Nucleolin, for example, binds to the HPV16 genome (Sato, et al., 2009) and has also been suggested to play a role in replication of HSV-1 DNA (Callé, et al., 2008). Furthermore, nucleolin is required for viral DNA synthesis and efficient virus production in human cytomegalovirus (HCMV)-infected cells (Strang, et al., 2010). The RNA helicase DDX5 is required for SARS coronavirus replication (Chen, et al., 2009), and is also involved in the replication of Hepatitis Virus C (HCV) (Goh, et al., 2004). The poly(rC)-binding protein (PCBP2, or hnRNP-E2) directs HCV RNA replication (Wang, et al., 2011), and is also involved in dengue virus replication (Rodenhuis-Zybert, 2010). Similarly, hnRNP-F, hnRNP-C and hnRNP-A1 are involved in the replication of the influenza A virus (Lee, et al., 2010), poliovirus (Brunner, et al., 2010), and HTLV-1, respectively (Kress, et al., 2005). As apoptin is itself a product of the Chicken Anemia Virus (CAV), the question arises whether these interactions might also be relevant for CAV replication. Coincidentally, CAV can only replicate in tumorigenic chicken cell lines, where it is localized in the nucleus, but does not replicate in normal chicken cell cultures (Noteborn, 2004).

Finally, the SV40 large T antigen was also identified in our analysis. Its presence in our experimental system is explained by the fact that this antigen was used to stably transform the cells used in our assay, as described in the Materials and Methods section. The oncogenic transformation potential of SV40 LT relies on its interaction with several tumor suppressors, including p53 and pRb (Ahuja, et al., 2005; Cheng, et al., 2009) and its expression has been shown to swiftly activate the DNA damage response (Boichuk, et al., 2010; Hein, et al., 2009). We have previously demonstrated (Zimmerman, et al., 2011a) that transient transformation of normal cells with SV40 LT is sufficient to activate apoptin, inducing its nuclear translocation, and subsequently, apoptosis. It is, however, the first time that we find an association, either directly or indirectly, between apoptin and SV40 LT.

Conclusion

The results presented here attest that the previously observed nucleolar localization of apoptin is not a random one, and that interaction with nucleolar proteins and R-chromatin is likely essential for apoptin's tumor-selective apoptosis-inducing activity. Importantly, nucleolin and nucleophosmin link apoptin to the DNA damage response; CDC5L and SET1 provide a connection to the regulation of mitosis, while the interactions with tubulin, nucleophosmin and nucleolin also corroborate the association of apoptin with the mitotic spindle apparatus. Furthermore, the interactions with proteins involved in ribosome biogenesis and RNA metabolism suggest a mechanism whereby apoptin shuts down the cellular factory and orchestrates its apoptotic effects from within the nucleolus.

A comparable mechanism has recently been demonstrated for the mouse Polo-like kinase 5, which also localizes to the nucleolus, and induces apoptosis in response to DNA damage (Andrysik, et al., 2010).

Recently, the pRb-regulated E2F family member E2F1 has been shown to induce DNA-damaged mediated apoptosis by transcriptional upregulation of the nucleolar protein RRP1B (Paik, et al., 2010). E2F1 itself is activated by Nur77 (Mu and Chang, 2003), which has been shown to participate in apoptin-induced apoptosis (Maddika, et al., 2005). Furthermore, apoptin has been shown to interact with DEDAF (Danen-van Oorschot, et al., 2004), which associates with DEDD in the nucleolus (Zheng, et al., 2001). It has indeed been reported that DEDD inhibits the Cdk1/cyclin B complex (Arai, et al., 2007), thereby arresting mitosis, and transferring the apoptotic signal to the nucleolus to shut off biosynthesis (Stegh, et al., 1998). Furthermore, apoptin has been shown to localize to the nucleus in response to DNA damage (Kucharski, et al., 2011), and induce G2/M arrest (Teodoro, et al., 2004). We therefore predict that apoptin's tumor-selective nuclear localization functions in recognizing activation of the DNA damage response, interfering with biosynthesis and arresting the mitotic cycle as a result, triggering apoptosis.

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References

Ahuja D, Sáenz-Robles M, and Pipas J (2005) SV40 large T antigen targets multiple cellular pathways to elicit cellular transformation. *Oncogene* 24, 7729-7745.

Ameyar-Zazoua M, Souidi M, Fritsch L, Robin P, Thomas A, Hamiche A et al. (2009) Physical and functional interaction between heterochromatin protein 1alpha and the RNA-binding protein heterogenous nuclear ribonucleoprotein U. *J Biol Chem* 284, 27974-27979.

Amin M, Matsunaga S, Uchiyama S and Fukui K (2008) Nucleophosmin is required for chromosome congression, proper mitotic spindle formation, and kinetochore-microtubule attachment in HeLa cells. *FEBS Lett* 582, 3839-3844.

Andrysik Z, Bernstein W, Deng L, Myer D, Li Y, Tischfield J et al. (2010) The novel mouse Polo-like kinase 5 responds to DNA damage and localizes in the nucleolus. *Nucleic Acids Res* 38, 2931-2943.

Arai S, Miyake K, Voit R, Nemoto S, Wakeland E, Grummt I et al. (2007) Death-effector domain-containing protein DEDD is an inhibitor of mitotic Cdk1/cyclin B1. *Proc Natl Acad Sci U S A* 104, 2289-2294.

Backendorf C, Visser A, De Boer A, Zimmerman R, Visser M, Voskamp P et al. (2009) Apoptin: Therapeutic potential of an early sensor of carcinogenic transformation. *Annu Rev Pharmacol Toxicol* 48, 143-169.

Balasubramani M, Day B, Schoen R and Getzenberg R (2006) Altered expression and localization of creatine kinase B, heterogeneous nuclear ribonucleoprotein F, and high mobility group box 1 protein in the nuclear matrix associated with colon cancer. *Cancer Res* 66, 763-769.

Berglund F and Clarke P (2009) hnRNP-U is a specific DNA-dependent protein kinase substrate phosphorylated in response to DNA double-strand breaks. *Biochem Biophys Res Commun* 381, 59-64.

Bernstein H and Coughlin S (1998) A mammalian homolog of fission yeast Cdc5 regulates G2 progression and mitotic entry. *J Biol Chem* 273, 4666-4671.

Bertwistle D, Sugimoto M and Sherr C (2004) Physical and functional interactions of the Arf tumor suppressor protein with nucleophosmin/B23. *Mol Cell Biol* 24, 985-996.

Boichuk S, Hu L, Hein J and Gjoerup O (2010) Multiple DNA damage signaling and repair pathways deregulated by simian virus 40 large T antigen. *J Virol* 84, 8007-8020.

Boisvert F, van Koningsbruggen S, Navascués J and Lamond A (2007) The multifunctional nucleolus. *Nat Rev Mol Cell Biol* 8, 574-585.

Boukakis G, Patrinou-Georgoula M, Lekarakou M, Valavanis C and Guialis A (2010) Deregulated expression of hnRNP A/B proteins in human non-small cell lung cancer: parallel assessment of protein and mRNA levels in paired tumour/non-tumour tissues. *BMC Cancer* 10, 434.

Boulon S, Westman B, Hutten S, Boisvert F and Lamond A (2010) The nucleolus under stress. *Mol Cell* 40, 216-227.

Brunner J, Ertel K, Rozovics J and Semler B (2010) Delayed kinetics of poliovirus RNA synthesis in a human cell line with reduced levels of hnRNP C proteins. *Virology* 400, 240-247.

Callé A, Ugrinova I, Epstein A, Bouvet P, Diaz J and Greco A (2008) Nucleolin is required for an efficient herpes simplex virus type 1 infection. *J Virol* 82, 4762-4773.

Canela N, Rodriguez-Vilarrupla A, Estanyol J, Diaz C, Pujol, M, Agell N et al. (2003) The SET protein regulates G2/M transition by

modulating cyclin B-cyclin-dependent kinase 1 activity. *J Biol Chem* 278, 1158-1164.

Chang K, Yang P, Lai H, Yeh T, Chen T and Yeh C (2006) Identification of a novel actin isoform in hepatocellular carcinoma. *Hepatol Res* 36, 33-39.

Chaudhury A, Hussey G, Ray P, Jin G, Fox P and Howe P (2010) TGF-β-mediated phosphorylation of hnRNP E1 induces EMT via transcript-selective translational induction of Dab2 and ILEI. *Nat Cell Biol* 12, 286–293.

Chen J, Chen W, Poon K, Zheng B, Lin X, Wang Y et al. (2009) Interaction between SARS-CoV helicase and a multifunctional cellular protein (Ddx5) revealed by yeast and mammalian cell two-hybrid systems. *Arch Virol* 154, 507-512.

Chen M, Zhang J and Manley J (2010) Turning on a fuel switch of cancer: hnRNP proteins regulate alternative splicing of pyruvate kinase mRNA. *Cancer Res* 70, 8977-8980.

Chen S, Maya-Mendoza A, Zeng K, Tang C, Sims P, Loric J et al. (2009) Interaction with checkpoint kinase 1 modulates the recruitment of nucleophosmin to chromatin. *J Proteome Res* 8, 4693-4704.

Cheng J, DeCaprio J, Fluck M and Schaffhausen B (2009) Cellular transformation by Simian Virus 40 and Murine Polyoma Virus T antigens. *Semin Cancer Biol* 19, 218-228.

Clark E, Coulson A, Dalgliesh C, Rajan P, Nicol S, Fleming S et al. (2008) The RNA helicase p68 is a novel androgen receptor coactivator involved in splicing and is overexpressed in prostate cancer. *Cancer Res* 68, 7938-7946.

Colombo E, Bonetti P, Denchi E, Martinelli P, Zamponi R, Marine J et al. (2005) Nucleophosmin is required for DNA integrity and p19Arf protein stability. *Mol Cell Biol* 25, 8874-8886.

Cui H, Wu F, Sun Y, Fan G and Wang Q (2010) Up-regulation and subcellular localization of hnRNP A2/B1 in the development of hepatocellular carcinoma. *BMC Cancer* 10, 356.

Danen-van Oorschot A, Fischer D, Grimbergen J, Klein B, Zhuang S-M, Falkenburg J et al. (1997) Apoptin induces apoptosis in human transformed and malignant cells but not in normal cells. *Proc Natl Acad Sci U S A* 94, 5843-5847.

Danen-van Oorschot A, Voskamp P, Seelen M, van Miltenburg M, Bolk M, Tait S et al. (2004) Human death effector domain-associated factor interacts with the viral apoptosis agonist Apoptin and exerts tumor-preferential cell killing. *Cell Death Differ* 11, 564-573.

Danen-van Oorschot A, Zhang Y-H, Leliveld S, Rohn J, Seelen M, Bolk M et al. (2003) Importance of nuclear localization of apoptin for tumor-specific induction of apotosis. *J Biol Chem* 278, 27729-27736.

de Hoog C, Foster L and Mann M (2004) RNA and RNA binding proteins participate in early stages of cell spreading through spreading initiation centers. *Cell* 117, 649–662.

Drygin D, Rice W and Grummt I (2010) The RNA Polymerase I Transcription Machinery: An Emerging Target for the Treatment of Cancer. *Annu Rev Parm Tox* 50, 131-156.

Eichler D and Craig N (1994) Processing of eukaryotic ribosomal RNA. *Prog Nucleic Acid Res Mol Biol* 49, 197-239.

Estanyol J, Jaumot M, Casanovas O, Rodriguez-Vilarrupla A, Agell N and Bachs O (1999) The protein SET regulates the inhibitory effect of p21(Cip1) on cyclin E-cyclin-dependent kinase 2 activity. *J Biol Chem* 274, 33161-33165.

Flynn R, Centore R, O'Sullivan R, Rai R, Tse A, Songyang Z et al. (2011) TERRA and hnRNPA1 orchestrate an RPA-to-POT1 switch on telomeric single-stranded DNA. *Nature* 471, 532-536.

Foltz D, Jansen L, Black B, Bailey A, Yates J 3rd,and Cleveland D (2006) The human CENP-A centromeric nucleosome-associated complex. *Nat Cell Biol* 8, 458-469.

Fousteri M, Vermeulen W, van Zeeland A and Mullenders L (2006) Cockayne syndrome A and B proteins differentially regulate recruitment of chromatin remodeling and repair factors to stalled RNA polymerase II in vivo. *Mol Cell* 23, 471-482.

Ginisty H, Sicard H, Roger B and Bouvet P (1999) Structure and functions of nucleolin. *J Cell Sci* 112, 761-772.

Goh E, Pardo O, Michael N, Niewiarowski A, Totty N, Volkova D et al. (2010) Involvement of heterogeneous ribonucleoprotein F in the regulation of cell proliferation via the mammalian target of rapamycin/S6 kinase 2 pathway. *J Biol Chem* 285, 17065-17076.

Goh P, Tan Y, Lim S, Tan Y, Lim S, Fuller-Pace F et al. (2004) Cellular RNA helicase p68 relocalization and interaction with the hepatitis C virus (HCV) NS5B protein and the potential role of p68 in HCV RNA replication. *J Virol* 78, 5288-5298.

Guha M, Pan H, Fang J and Avadhani N (2009) Heterogenous nuclear ribonucleoprotein A2 is a common transcriptional coactivator in the nuclear transcription response to mitochondrial respiratory stress. *Mol Cell Biol* 20, 4107-4119.

Han S, Tang Y and Smith R (2010) Functional diversity of the hnRNPs: past, present and perspectives. *Biochem J* 430, 379-392.

Hein J, Boichuk S, Wu J, Cheng Y, Freire R, Jat P et al. (2009) Simian virus 40 large T antigen disrupts genome integrity and activates a DNA damage response via Bub1 binding. *J Virol* 83, 117-127.

Ishikawa F, Matunis M, Dreyfuss G and Cech T (1993). Nuclear proteins that bind the pre-mRNA 3' splice site sequence r(UUAG/G) and the human telomeric DNA sequence d(TTAGGG)n. *Mol Cell Biol* 13, 4301-4310.

Ito S, Tan LJ, Andoh D, Narita T, Deki M, Hirano Y et al. (2010) MMXD, a TFIIH-independent XPD-MMS19 protein complex involved in chromosome segregation. *Cell* 39, 632-640.

Jauliac S, López-Rodriguez C, Shaw L, Brown L, Rao A and Toker A (2002) The role of NFAT transcription factors in integrin-mediated carcinoma invasion. *Nat Cell Biol* 4, 540-544.

Jing G, Xu D, Shi S, Li Q, Wang S, Wu F et al. (2011) Aberrant expression and localization of hnRNP-A2/B1 is a common event in human gastric adenocarcinoma. *J Gastroenterol Hepatol* 26, 108-115.

Jordan P, and Carmo-Fonseca M. (1998). Cisplatin inhibits synthesis of rRNA in vivo. *Nucleic Acids Res,* 26, 2831–2836.

Klein B, Pastink A, Odijk H, Westerveld A and Van der Eb A (1990) Transformation and immortalization of diploid xeroderma pigmentosum fibroblasts. *Exp Cell Res* 191, 256-262.

Kress E, Baydoun H, Bex F, Gazzolo L and Duc Dodon M (2005) Critical role of hnRNP A1 in HTLV-1 replication in human transformed T lymphocytes. *Retrovirology 2,* 8.

Kucharski T, Gamache I, Gjoerup O and Teodoro J (2011) DNA Damage Response Signaling Triggers Nuclear Localization of the Chicken Anemia Virus Protein Apoptin. *J Virol*, *Epub ahead of print*.

Kurki S, Peltonen K, Latonen L, Kiviharju T, Ojala P, Meek D et al. (2004) Nucleolar protein NPM interacts with HDM2 and protects tumor suppressor protein p53 from HDM2-mediated degradation. *Cancer Cell* 5, 465-475.

LaBranche H, Dupuis S, Ben-David Y, Bani M, Wellinger R and Chabot B (1998) Telomere elongation by hnRNP A1 and a derivative that interacts with telomeric repeats and telomerase. *Nat Genet* 19, 199-202.

Lee J, Kim S, Pascua P, Song M, Baek Y, Jin X et al. (2010) Direct interaction of cellular hnRNP-F and NS1 of influenza A virus accelerates viral replication by modulation of viral transcriptional activity and host gene expression. *Virology* 397, 89-99.

Leliveld S, Dame R, Mommaas M, Koerten H, Wyman C, Danen-van Oorschot A, et al. (2003). Apoptin protein multimers form distinct higher-order nucleoprotein complexes with DNA. *Nucleic Acids Res,* 31, 4805-4813.

Leliveld S, Dame R, Rohn J, Noteborn M and Abrahams J (2004) Apoptin's functional N- and C-termini independently bind DNA. *FEBS Lett* 557, 155-158.

Leung J, Leitch A, Wood J, Shaw-Smith C, Metcalfe K, Bicknell L et al. (2011) SET nuclear oncogene associates with microcephalin/MCPH1 and regulates chromosome condensation. *J Biol Chem* 286, 21393-21400.

Li M, Makkinje A and Damuni Z (1996) The myeloid leukemia-associated protein SET is a potent inhibitor of protein phosphatase 2A. *J Biol Chem* 271, 11059-11062.

Li X, Liu Y, Wen Z, Li C, Lu H, Tian M et al. (2010). Potent anti-tumor effects of a dual specific oncolytic adenovirus expressing apoptin in vitro and in vivo. *Mol Cancer* 9, 10.

Liu H and Yung B (1999) In vivo interaction of nucleophosmin/B23 and protein C23 during cell cycle progression in HeLa cells. *Cancer Let* 144, 45-54.

Liu X, Bera T, Liu L and Pastan I (2009) A primate-specific POTE-actin fusion protein plays a role in apoptosis. *Apoptosis* 14, 1237-1244.

Ma N, Matsunaga S, Morimoto A, Sakashita G, Urano T, Uchiyama S et al. (2011) The nuclear scaffold protein SAF-A is required for kinetochore-microtubule attachment and contributes to the targeting of Aurora-A to mitotic spindles. *J Cell Sci* 124, 394-404.

Ma N, Matsunaga S, Takata H, Ono-Maniwa R, Uchiyama S and Fukui K (2007) Nucleolin functions in nucleolus formation and chromosome congression. *J Cell Sci* 120, 2091-2105.

Ma Y, Peng J, Zhang P, Huang L, Liu W, Shen T et al. (2009) Heterogenous nuclear ribonucleoprotein A1 is identified as a potential biomarker for colorectal cancer based on differential proteomics technology. *J Proteome Res* 8, 4525-4535.

Maddika S, Booy E, Johar D, Gibson S, Ghavami S and Los M (2005) Cancer-specific toxicity of apoptin is independent of death receptors but involves the loss of mitochondrial membrane potential and the release of mitochondrial cell-death mediators by a Nur77-dependent pathway. *J Cell Sci* 118, 4485–4493.

Maddika S, Panigrahi S, Wiechec E, Wesselborg S, Fischer U, Schulze-Osthoff K et al. (2009) Unscheduled Akt-triggered activation of cyclin-dependent kinase 2 as a key effector mechanism of apoptin's anticancer toxicity. *Mol Cell Biol* 29, 1235-1248.

Maggi L J, Kuchenruether M, Dadey D, Schwope R, Grisendi S, Townsend, R et al. (2008) Nucleophosmin serves as a rate-limiting nuclear export chaperone for the mammalian ribosome. *Mol Cell Biol* 28, 7050-7065.

Montanaro L, Treré D and Derenzini M (2008) Nucleolus, ribosomes, and cancer. *Am J Pathol* 173, 301-310.

Moss T and Stefanovsky V (2002) At the center of eukaryotic life. *Cell* 109, 545-548.

Mu X and Chang C (2003) TR3 orphan nuclear receptor mediates apoptosis through up-regulating E2F1 in human prostate cancer LNCaP cells. *J Biol Chem* 278, 42840-42845.

Noteborn M (2004). Chicken anemia virus induced apoptosis: underlying molecular mechanisms. *Vet Microbiol* 98, 89-94.

Okuda M, Horn H, Tarapore P, Tokuyama Y, Smulian A, Chan P et al. (2000) Nucleophosmin/B23 is a target of CDK2/cyclin E in centrosome duplication. *Cell* 103, 127-140.

Okuwaki M, Tsujimoto M and Nagata K (2002) The RNA binding activity of a ribosome biogenesis factor, nucleophosmin/B23, is modulated by phosphorylation with a cell cycle-dependent kinase and by association with its subtype. *Mol Biol Cell* 13, 2016-2030.

Paik J, Wang B, Liu K, Lue J and Lin W (2010) Regulation of E2F1-induced apoptosis by the nucleolar protein RRP1B. *J Biol Chem* 285, 6348-6363.

Piacentini L, Fanti L, Negri R, Del Vescovo V, Fatica A, Altieri F and Pimpinelli S (2009) Heterochromatin protein 1 (HP1a) positively regulates euchromatic gene expression through RNA transcript association and interaction with hnRNPs in Drosophila. *PLosOne Genet*, e1000670.

Pianese G (1896) Beitrag zur Histologie und Aetiologie der Carcinoma. Histologische und experimentelle Untersuchungen. *Beitr Pathol Anat Allgem Pathol* 142, 1-193.

Pianta A, Puppin C, Franzoni A, Fabbro D, Di Loreto C, Bulotta S et al. (2010) Nucleophosmin is overexpressed in thyroid tumors. *Biochem Biophys Res Commun* 397, 499-504.

Pillai M, Chacko P, Kesari L, Jayaprakash P, Jayaram H and Antony A (2003) Expression of folate receptors and heterogeneous nuclear ribonucleoprotein E1 in women with human papillomavirus mediated transformation of cervical tissue to cancer. *J Clin Pathol* 56, 569–574.

Poon I, Oro C, Dias M, Zhang J and Jans D (2005) Apoptin nuclear accumulation is modulated by a CRM1-recognized nuclear export signal that is active in normal but not in tumor cells. *Cancer Res* 65, 7059-7064.

Rappsilber J, Mann M and Ishihama Y (2007) Protocol for micro-purification, enrichment, pre-fractionation and storage of peptides for proteomics using StageTips. *Nat Protoc* 8, 1896-1906.

Rodenhuis-Zybert I, Wilschut J and Smit J (2010). Dengue virus life cycle: viral and host factors modulating ingfectivity. *Cell Mol Life Sci* 67, 2773-2786.

Rohn J, Zhang Y-H, Aalbers R, Otto N, den Hertog J, Henriquez N et al. (2002) A tumor-specific kinase activity regulates the viral death protein apoptin. *J Biol Chem* 277, 50820-50827.

Rousselet A (2009) Inhibiting Crm1 causes the formation of excess acentriolar spindle poles containing NuMA and B23, but does not affect centrosome numbers. *Biol Cell* 101, 679-693.

Roychoudhury P, Paul R, Chowdhury R and Chaudhuri K (2007) HnRNP E2 is down-regulated in human oral cancer cells and the over-expression of hnRNP E2 induces apoptosis. *Mol Carcinog* 46, 198-207.

Sato H, Kusumoto-Matsuo R, Ishii Y, Mori S, Nakahara T, Shinkai-Ouchi F et al. (2009) Identification of nucleolin as a protein that binds to human papillomavirus type 16 DNA. *Biochem Biophys Res Commun* 387, 525-530.

Schiffner S, Zimara N, Schmid R and Bosserhoff A (2011) p54nrb is a new regulator of progression of malignant melanoma. *Carcinogenesis* 32, 1176-1182.

Srivastava M and Pollard H (1999) Molecular dissection of nucleolin's role in growth and cell proliferation: new insights. *FASEB J* 13, 1911-1922.

Stegh A S (1998) DEDD, a novel death effector domain-containing protein, targeted to the nucleolus. *EMBO J* 17, 5974-5986.

Strang B, Boulant S and Coen D (2010) Nucleolin associates with the human cytomegalovirus DNA polymerase accessory subunit UL44 and is necessary for efficient viral replication. *J Virol 84,* 1771-1784.

Sun J, Yan Y, Wang X, Liu X, Peng D, Wang M et al. (2009) PTD4-apoptin protein therapy inhibits tumor growth in vivo. *Int J Cancer* 124, 2973-2981.

Tauler J, Zudaire E, Liu H, Shih J and Mulshine J (2010) hnRNP A2/B1 modulates epithelial-mesenchymal transition in lung cancer cell lines. *Cancer Res 70*, 7137-7147.

Tembe V and Henderson B (2007) Protein trafficking in response to DNA damage. *Cell Signal* 19, 1113-1120.

Teodoro J, Heilman D, Parker A, and Green, M. (2004) The viral protein Apoptin associates with the anaphase-promoting complex to induce G2/M arrest and apoptosis in the absence of p53. *Genes Dev* 18, 1952-1957.

Vera J, Estanyol J, Canela N, Llorens F, Agell N, Itarte E et al. (2007) Proteomic analysis of SET-binding proteins. *Proteomics* 7, 578-587.

Wagstaff K and Jans D (2009) Nuclear drug delivery to target tumour cells. *Eur J Pharmacol 625*, 174-180.

Wang G, Zheng Y, Che X, Wang X, Zhao J, Wu K et al. (2009) Upregulation of human DNA binding protein A (dbpA) in gastric cancer cells. *Acta Pharmacol Sin* 30, 1436-1442.

Wang L, Jeng K and Lai M (2011) Poly(C)-binding protein 2 interacts with sequences required for viral replication in the hepatitis C virus (HCV) 5' untranslated region and directs HCV RNA replication through circularizing the viral genome. *J Virol* 85, 7954-7964.

Wardleworth B and Downs J (2005) Spotting new DNA damage-responsive chromatin binding proteins. *Biochem J* 388, e1-2.

Wessel D and Flugge U (1984) A method for the quantitative recovery of protein in dilute solution in the presence of detergents and lipids. *Anal Biochem 138* 141-143.

Wiśniewski J, Zougman A, Nagaraj N and Mann M (2009) Universal sample preparation method for proteome analysis. *Nat Methods* 6, 359-362.

Yao Z, Duan S, Hou D, Wang, W, Wang G, Liu Y et al. (2010) B23 acts as a nucleolar stress sensor and promotes cell survival through its dynamic interaction with hnRNPU and hnRNPA1. *Oncogene* 29, 1821-1834.

Zhang N, Kaur R, Akhter S and Legerski R (2009) Cdc5L interacts with ATR and is required for the S-phase cell-cycle checkpoint. *EMBO Rep 10,* 1029-1035.

Zhang N, Kaur R, Lu X, Shen X, Li L and Legerski R (2005) The Pso4 mRNA splicing and DNA repair complex interacts with WRN for processing of DNA interstrand cross-links. *J Biol Chem* 280, 40559-40567.

Zhang Y-H (2004a). Activation of apoptin, the tumor-specific viral death effector. PhD Thesis, Leiden University

Zhang Y-H, Kooistra K, Pietersen A, Rohn JL and Noteborn, M. (2004b). Activation of the tumor-specific death effector apoptin and its kinase by an N-terminal determinant of simian virus 40 large T antigen. *J Virol* 78, 9965-9976.

Zhang Y and Lu H (2009) Signaling to p53: ribosomal proteins find their way. *Cancer Cell* 16, 369-377.

Zheng L, Schickling O, Peter M and Lenardo M (2001) The death effector domain-associated factor plays distinct regulatory roles in the nucleus and cytoplasm. *J Biol Chem* 276, 31945–31952.

Zimmerman R, Peng D, Zhang Y-H, Danen-Van Oorschot A, Qu S, Backendorf C and Noteborn, M. (2011a). Nuclear PP2A inactivation is a crucial step in triggering apoptin-induced tumor-selective cell killing. Submitted for publication.

Zimmerman RME, Sun J, Tian J, de Smit M, Danen-van Oorschot AA, Rotman M et al. (2011b). Cellular partners of the apoptin-interacting protein 3. Submitted for publication.

Chapter 6

PP2A inactivation is a crucial step in triggering apoptin-induced tumor-selective cell killing

Rhyenne Zimmerman[1,*], Dongjun Peng[1,2,*], Henriette Lanz[1], Ying-Hui Zhang[1], Astrid Danen-Van Oorschot[1], Shen Qu[2], Claude Backendorf[1] and Mathieu Noteborn[1,2,¶]

[1]Department of Molecular Genetics, Leiden Institute for Chemistry, Leiden University, Leiden, The Netherlands, [2]Department of Biochemistry and Molecular Biology, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China

¶Corresponding author: M.H.M. Noteborn, Department of Molecular Genetics, Graduate School of Chemistry, Leiden University, Einsteinweg 55, 2333 CC Leiden, The Netherlands. Tel: +31 71 5274544; Fax: +31 71 5274430; E-mail: m.noteborn@chem.leidenuniv.nl

*contributed equally to the manuscript

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Abbreviations: apoptin, apoptosis inducing protein; AKIP1, A-kinase interacting protein; BCA3, breast cancer-associated gene 3; DAPI, 2,4-diamidino-2-phenylindole; NLS, nuclear localization signal; PKA, protein kinase A; PP2A, protein phosphatase 2A; Rb, Retinoblastoma protein; RNAi, RNA interference; RSV, Rous sarcoma virus; SV40 LT, SV40 large T antigen; SV40 ST, SV40 small t antigen

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Abstract

Apoptin harbors tumor-selective characteristics, making it a potentially safe and effective anticancer agent. Apoptin becomes phosphorylated and induces apoptosis in a large panel of human tumor but not normal cell lines. Here, we explored minimal cellular factors required for the activation of apoptin. Flag-apoptin was introduced into normal fibroblasts together with the transforming SV40 LT and ST antigens. We found that expression of nuclearly located SV40 ST in normal cells was sufficient to induce phosphorylation of apoptin. Mutational analysis showed that C103S mutation within the protein phosphatase 2A (PP2A)-binding domain of ST counteracted this effect. Knock-down of the ST-interacting PP2A B56γ subunit in normal fibroblasts mimicked the effect of nuclear ST expression, resulting in induction of apoptin phosphorylation. The same effect was observed upon down-regulation of the PP2A B56δ subunit, which is targeted by PKA. Apoptin interacts with the PKA-associating protein BCA3 (AKIP1), and inhibition of PKA in tumor cells by treatment with H89 increased the phosphorylation of apoptin, whereas the PKA activator cAMP partially reduced it. We therefore conclude that inactivation of PP2A, particularly of the B56γ and B56δ subunits, is a crucial step in triggering apoptin-induced tumor-selective cell death.

Introduction

Tumor formation occurs due to a complicated set of processes roughly based on enhanced survival and limited cell death activities (Wenner, 2010). Remarkably, a set of viral and cellular proteins has been found to selectively induce cell death in tumor cells (Bruno, et al., 2009). Among these proteins is the avian virus protein apoptin, which has been shown to induce p53-independent apoptosis in a broad spectrum of human transformed cells (Backendorf, et al., 2008). Recent preclinical studies demonstrated the therapeutic potential of apoptin as a safe and efficient anticancer agent (Grimm and Noteborn, 2010). Therefore, it is of interest to study the mechanisms underlying apoptin-induced apoptosis, particularly the 'switch' responsible for its activation during oncogenic transformation.

SV40 T antigens are known to be involved in oncogenic transformation through interference with many cellular processes (Pipas, 2009). Distinct domains on LT that bind and inactivate tumor suppressors p53 and Rb, have long been known to play crucial roles in tumor formation (Eichhorn, et al., 2009). The SV40 ST protein enforces transformation of normal cells via negative effects on the protein phosphatase 2A (Sablina, et al., 2010).

This feature is in accordance with the reduced PP2A levels found in various human tumor cell types (Sablina and Hahn, 2008), and accumulating evidence supporting major tumor suppressive roles for PP2A (Westermarck and Hahn, 2008). Another major cellular regulator implicated in carcinogenesis is protein kinase A (PKA) (Naviglio, et al., 2009). Its targets include PP2A (Usui, et al., 1998), and for instance, the PKA interacting protein Breast Cancer-associated gene 3 (BCA3) is known to be highly expressed in breast and prostate cancer cells compared to the normal surrounding tissue (Kitching, et al., 2003).

In this study, we investigated the minimal steps leading to the activation of apoptin upon malignant transformation. We previously showed that

transient expression of the SV40 large and small T antigens in normal human fibroblasts results in activation of apoptin, displaying all three of its characteristic features, namely phosphorylation, nuclear localization, and apoptosis (Zhang, et al., 2004). Here, we analyzed which domains of the SV40 large and/or small T antigens were responsible for this activity. In parallel, BCA3 was identified as an apoptin-interacting protein, and the effects of PKA on apoptin phosphorylation were examined. Analysis of both transformation-related pathways revealed that inactivation of PP2A is crucial for the activation of apoptin.

Materials and Methods

Cells and Cell culture

Human diploid foreskin F9 fibroblasts, isolated from neonatal foreskin, were obtained in the late 1980's from Dr. M. Ponec (Dept. Dermatology, Leiden University Medical Center). Cells were batch-frozen after careful morphological inspection. At subsequent passages cells were regularly screened for their typical fibroblast-like morphological appearance. All cells used were below passage 15 and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% newborn calf serum, 100 U/ml penicillin and 100 µg/ml streptomycin (Invitrogen, Breda, the Netherlands). The human Saos-2 osteosarcoma and the HeLa cervical carcinoma cell lines were purchased from the American Type Culture Collection (ATCC) and cultured in the same medium as mentioned above. Cultures were regularly tested to ensure the absence of *Mycoplasma* infection. Cell morphology was regularly monitored to control the absence of cross-contamination. The sensitivity to apoptin is characteristic of the various cell types used (Danen-van Oorschot, et al., 1997) and is regularly assessed (see below).

DNA plasmids

The DNA sequence encoding apoptin was synthesized by BaseClear (Leiden, the Netherlands) according to the apoptin sequence published by Noteborn et al. (1991), and cloned into the mammalian expression vector pcDNA3.1(+)

(Invitrogen). The oligonucleotide fragment encoding the Flag-tag (Invitrogen) was inserted to create the pcDNA-Flag-apoptin plasmid encoding apoptin fused with a Flag-tag at its N-terminus.

Plasmid pRSV-TN136 encoding the first 136 N-terminal amino acids of SV40 LT, including the region coding for SV40 ST, was a kind gift from Dr. J.M. Pipas (Department of Biological Sciences, University of Pittsburgh, Pittsburgh, PA, USA). From pRSV-TN136, we generated the pcDNA-LT136/ST plasmid, which encodes the LT136 (The N-terminally truncated LT fragment containing the first 1-136 aa) and full length ST. pcDNA-LT136, encoding LT136 only, was constructed by introducing an intron deletion disabling ST expression (Yamashita, et al., 1990). pcDNA expression vectors encoding only ST sequences were derived from pRSV-ST. pcDNA-ST encodes for ST; pcDNA-NLS-ST contains a ST fused to a nuclear localization signal (NLS-ST) and pcDNA-NLS-ST(C103S) encodes the N-terminal NLS-ST fusion protein containing the C103S mutation within the PP2A binding site (Gjoerup, et al., 2001).

The sequences encoding the N-terminal 136 aa of LT together with either full-length ST or the C103S ST-mutant were cloned into pEXPR-IBA105 vector to generate pEXPR-IBA105-LT136/ST and pEXPR-IBA105-LT136/STC103S respectively. These plasmids expressed Strep-tagged LT136/ST or LT136/ST C103S proteins enabling interaction studies with PP2A (see below) (Schmidt and Skerra, 2007). pCEP-4HA-B56γ, encoding 4HA-tagged B56γ, was a kind gift from Dr. M. Mumby (University of Texas, USA).

Apoptin-interacting partners were obtained by yeast two-hybrid screening and verified by immunoprecipitation assays in mammalian cells, as previously described by Danen-Van Oorschot et al. (Danen-van Oorschot, et al., 2004)**.** Positive clones from the yeast two-hybrid screen were digested with Xho-I to generate cDNA fragments, and subcloned into pMT2SM-myc to provide the fragments with an in-frame N-terminal myc-tag. The cDNA

fragment encoding BCA3, including the myc-tag, was subsequently cloned into pcDNA.

Transfection Methods

We used transfection reagent DOTAP (N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium methylsulfate) (Danen-van Oorschot, et al., 1997) or AMAXA nucleofection technology in conjunction with cell type specific NucleofectorTM solution (Lonza, Cologne, Germany) (Rohn, et al., 2002) for DNA delivery into cells. When co-transfection or triple-transfection was performed, the ratio of each plasmid was 1:1 or 1:1:1 (in micrograms). In addition to the analysis by Western blot, the cells were seeded on several glass cover slips to allow parallel analysis at the single-cell level by immunofluorescence assay.

Western Blot analysis

Cells were lysed directly in Laemmli buffer (2% SDS, 10% Glycerol, 60mM Tris-Cl [pH6.8], 2% β-mercaptoethanol, 0.002% bromophenol blue). Cell lysates were separated by sodium dodecyl-sulfate-polyacrylamide gel electrophoresis, and electroblotted onto polyvinylidene difluoride membranes (Bio-Rad). Blots were then incubated with antibodies against phosphorylated-apoptin (α-108-P; Zhang, et al., 2004), Flag-apoptin (α-Flag M2, Sigma-Aldrich), SV40 LT (PAb416, Pab419, Calbiochem), SV40 ST (Pab280, Calbiochem), PP2A Aα (C-20, Santa Cruz), PP2A B56γ (α-B56γ, kind gift from Dr. Marc Mumby, Health Science Center, University of Texas, Texas, USA), PP2A B56δ (α-B56δ, Santa Cruz Biotechnology), myc-tagged BCA3 (α-myc, BD Biosciences), and actin (α-actin, Santa Cruz). Horseradish peroxidase-conjugated goat antibody against rabbit or mouse immunoglobulin G, or rabbit antibody against goat immunoglobulin G (Sigma-Aldrich) was used as secondary antibody for signal detection by enhanced chemiluminescence.

Protein interaction assays

Detection of a possible interaction of ST or ST-C103S mutant protein with PP2A in human HeLa cells was performed as follows. Twenty-four hours after DNA transfection, cells were washed twice with phosphate buffered saline and harvested in ice-cold mild lysis buffer (50mM Tris [pH 7.5], 5mM EDTA, 250mM NaCl, 0.1% Triton X-100, 5 mM NaF, 1mM Na_3VO_4, 20mM beta-glycerolphosphate, and Protease Inhibitor Cocktail (Roche), followed by incubation on ice for 30 min. The supernatant of the lysates was prepared by centrifugation at 13,000 × g and 4°C for 30 min. Strep-tagged proteins and their interacting proteins were captured using the One-strep kit (IBA, Germany) according to the manufacturer's protocol, and resolved on sodium dodecyl sulfate-polyacrylamide gel, followed by Western blotting analysis with appropriate antibodies.

RNA interference assay

For human PP2A B56γ, the target sequence was: 5'-GATGAACCAACGTTAGAAG-3'; for PP2A B56δ two sequences were targeted (1): 5' GTGTGTCTCTAGCCCCCAT 3' (Arnold, et al., 2006) and (2) 5' GACCATTTTGCATCGCATC 3' (van Kanegan and Strack 2009) (data not shown). The pSUPER vector was designated for shRNA plasmid constructions (Boutros and Ahringer, 2008). The amplification and purification of plasmids were performed as specified by manufacturer's instruction (GeneService, Cambridge, UK). Cells transfected with shRNA plasmids were lysed at 24-48 h after transfection, and then analyzed by Western blot assay as described above.

Immunofluorescence assay

Cells were grown on glass cover slips. At indicated time points after transfection, cover slips were first washed once with phosphate buffered saline, and subsequently fixed with methanol/acetone (50%/50%) for 5-10 min at room temperature. After air-drying, the slides were used for immunocytochemical staining or stored at -20°C for further analysis. Immunocytochemical staining was carried out as described by Danen-Van

Oorschot et al. (2004). The following antibodies were used: α-108-P, a rabbit polyclonal antibody recognizing phosphorylated apoptin at T108 and α-Flag, a mouse monoclonal antibody recognizing Flag-apoptin. SV40 proteins were visualized with PAb416, a mouse monoclonal antibody recognizing the epitope residing in amino acids 83-128 of LT and non-reactive with ST or PAb280, a mouse monoclonal antibody against the C terminus of ST. The fluorescein isothiocyanate (FITC) or rhodamine conjugated goat antibodies (Jackson ImmunoResearch Laboratories) were used as secondary antibodies. Nuclei were stained with 2,4-Diamidino-2-phenylindole (DAPI) and apoptosis was assessed according to characteristic morphological changes (Danen-van Oorschot et al., 1997).

PKA inhibition and stimulation

Saos-2 cells were transfected with pcDNA-Flag-apoptin and incubated 24 hours post-transfection with 10μM H89 (Millipore) for 1 hour (PKA inhibition), or 30 minutes with H89 followed by incubation with 1mM cAMP (Sigma) for another 30 minutes (PKA stimulation). Cells were then lysed in Laemmli buffer and the cell lysates analyzed for apoptin phosphorylation by Western blotting.

Results

Transient expression of N-terminal SV40 LT136/ST activates apoptin in normal human fibroblasts

Apoptin activity can be induced in normal cells by transient expression of transforming SV40 antigens (Zhang, et al., 2004). Here, we examined the minimal SV40 domains responsible for this effect. Expression of the SV40 LT136/ST DNA sequence results, due to alternative splicing, in 2 proteins: LT136, comprising the N-terminal 136 a.a. of LT, and the entire ST protein, consisting of 174 amino acids (Figure 6.1A) (Srinivasan, et al., 1997). LT136 contains a DNA J domain, an Rb-binding site and a nuclear localization signal (NLS). ST shares its DNA J domain with LT136, but has a unique C-terminal domain encompassing a PP2A binding site (Pipas, 2009).

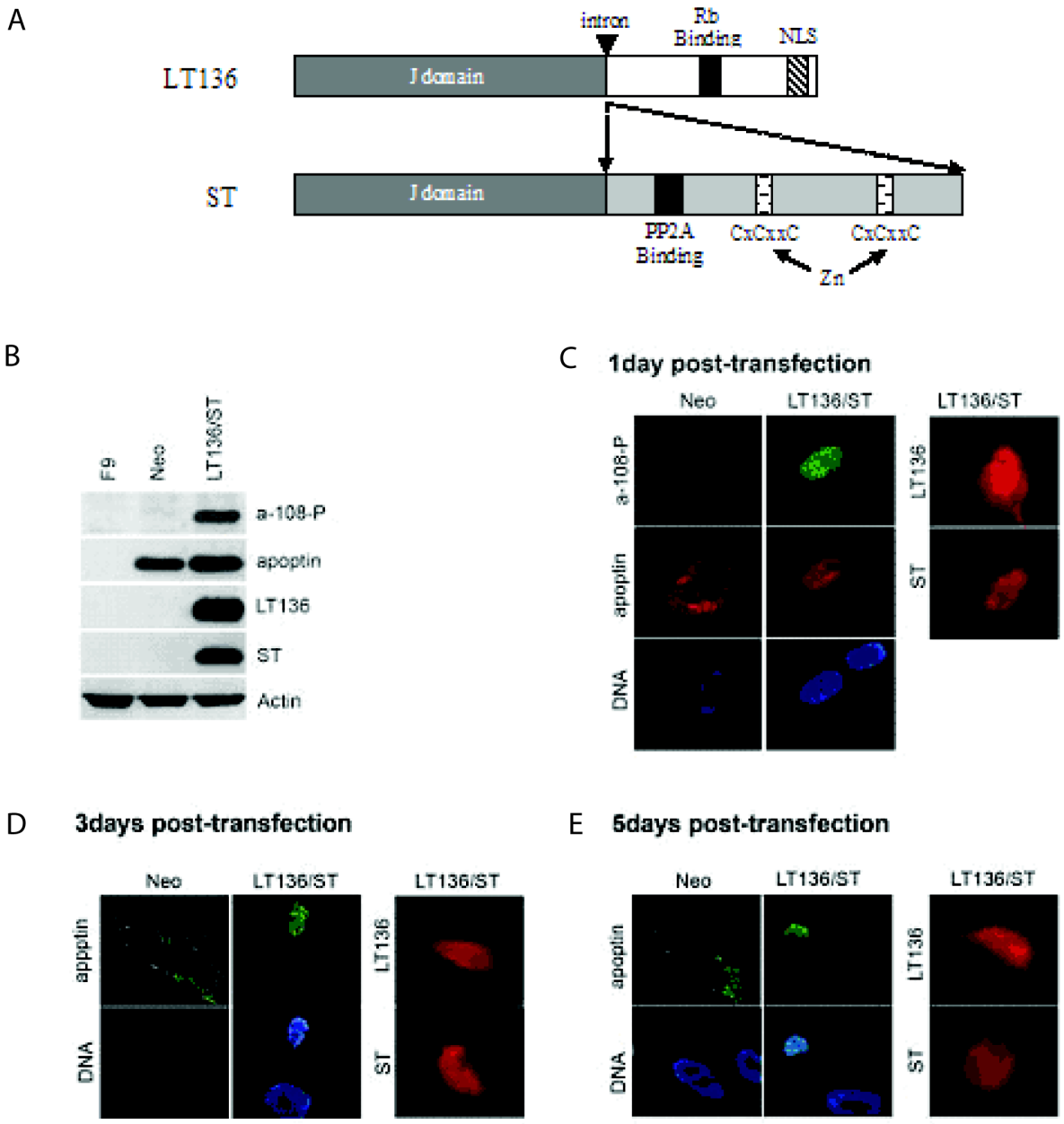

Figure 6.1 Transient expression of N-terminal determinants of SV40 T antigens activate apoptin. **A.** Schematic representation of the domains in SV40 LT136/ST. The J domain (a.a. 1-82) is identical in both LT136 and ST. The Rb binding domain and nuclear localization signal (NLS) in LT136 are also shown. ST is expressed by differential splicing and has a unique C-terminus, which contains the PP2A binding domain. **B.** Human Fibroblasts (F9) were co-transfected with plasmids pcDNA-Flag-apoptin and pcDNA-LT136/ST (LT136/ST) or pcDNA-neo (neo) by AMAXA nucleofector transfection. Twenty-four hours post-transfection, cells were lysed for Western blot analysis with the indicated antibodies. Mock-transfected F9 cells were used as control. Antibody α-108-P specifically recognizes phospho-apoptin at its Thr108. Flag-apoptin, LT and ST show the respective total protein amounts in the transfected cells. Actin was used as loading control. **C-E.** Cells were fixed for immunofluorescence analysis at each given time point after transfection and then stained with the indicated antibodies by indirect immunofluorescence assay. Scale bar = 20 μm.

In normal human foreskin fibroblasts, co-transfection of the SV40 LT136 and ST proteins with Flag-tagged apoptin resulted in the latter's phosphorylation (Figure 6.1B). Immunofluorescence analysis of F9 cells expressing all three proteins showed that Flag-apoptin became nuclear already 1 day after transfection. Nuclear Flag-apoptin was also shown to be phosphorylated (Figure 6.1C), and 3 and 5 days post transfection, apoptin was shown to induce apoptosis (Figure 6.1D, E). In contrast, F9 fibroblasts expressing apoptin alone contained mainly cytoplasmic Flag-apoptin, which was, as expected, not phosphorylated (Alvisi, et al., 2006; Rohn, et al., 2004) and did not induce apoptosis (Figure 6.1C-E).

Our results clearly reveal that transient expression of LT136 and ST proteins in human F9 fibroblasts results in tumor-selective activation of apoptin, providing us with the possibility to explore which domains and respective cellular targets of LT136/ST were responsible for this activity.

Nuclearly localized ST triggers apoptin activation

Next, we examined whether expression of either LT136 or ST protein alone was sufficient to activate apoptin phosphorylation. F9 fibroblasts were co-transfected with plasmids encoding Flag-tagged apoptin and either one of the following: a) pcDNA-LT136, encoding LT136, b) pcDNA-ST, encoding ST, c) pcDNA-LT136/ST, where both LT136 and ST are produced via alternative splicing, or d) pcDNA-neo, as a negative control. In each experiment, activation of apoptin was assayed by its phosphorylation at position T108, assessed by means of Western blotting. Expression of LT136 alone did not trigger apoptin phosphorylation, whereas expression of ST clearly induced apoptin phosphorylation, albeit at a low level (Figure 6.2). Co-transfection of Flag-tagged apoptin with ST fused to an artificial nuclear location signal (NLS-ST) increased the level of apoptin phosphorylation significantly (Figure 6.3B).

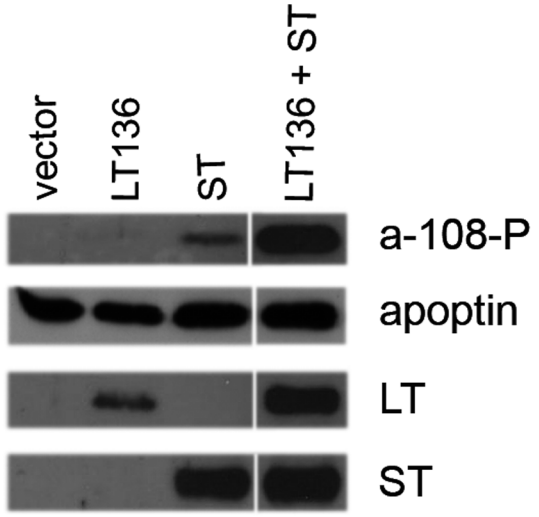

Figure 6.2 Nuclear targeting of SV40 ST activates apoptin. In normal human fibroblasts, apoptin was co-transfected with vector only (Neo), LT136, ST, or both LT136 and ST. Twenty-four hours post-transfection, cells were lysed for Western blot analysis with the antibodies indicated.

C103S mutation within PP2A-binding domain of ST disables activation of apoptin

Besides its J domain, shared with LT136, ST contains a unique site for the binding and inactivation of PP2A. This domain has been shown to contribute to cellular transformation (Pipas, 2009). A single amino-acid mutation C103S within the ST protein drastically diminishes the interaction of ST with PP2A (Figure 6.3A) and its transforming capacity (Fahrbach, et al., 2008). Therefore, we studied the effect of the C103S mutation within the PP2A binding site on the activation of apoptin by (nuclear) ST in normal human cells.

F9 cells were analyzed for phosphorylation of Flag-apoptin upon co-expression with ST, NLS-ST or NLS-ST(C103S) protein. Figure 6.3B shows that expression of NLS-ST clearly induced apoptin phosphorylation. Introduction of the NLS-ST(C103S) mutation completely abolished this induction, although apoptin protein was expressed at a similar level. These results suggest that ST interaction with PP2A is crucial to apoptin activation, and that inactivation of PP2A by ST might be sufficient to activate apoptin.

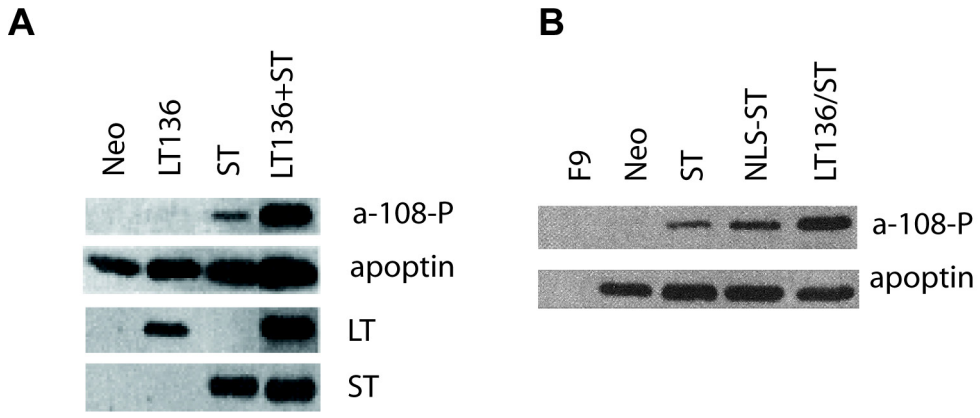

Figure 6.3 Apoptin activation via inhibition of PP2A by ST. **A.** PP2A binding to ST is abolished by C103S mutation. LT136 and ST with or without the C103S mutation (LT136/ST or LT136/ST(C103S) were fused with a Strep-tag at their N-terminus. Cell lysates were prepared at 24h post-transfection for protein-protein interaction assays as indicated in Material and Methods. The final elutions were analyzed by Western Blot with antibodies against PP2Aα subunit, LT and ST, respectively. Actin was taken as equal loading control. The first lane (input control) indicated total amount of endogenous proteins in direct cell lysates. **B.** pcDNA-Flag-apoptin was co-transfected with plasmids encoding the indicated proteins or vector DNA into F9 primary fibroblasts. Western blot assays were performed with the indicated antibodies at 24h post-transfection.

Knock-down of PP2A B56γ via RNAi activates apoptin phosphorylation in normal human fibroblasts

Two independent studies reported that ST interaction with PP2A resulted in the inhibition of the B56γ regulatory subunit, resulting in cellular transformation (Chen, et al., 2007; Cho, et al., 2007). Therefore, we examined whether down-regulation of B56γ via RNAi could trigger phosphorylation of apoptin in normal cells. Our shRNA sequence was verified to reduce ectopic expression of B56γ (Figure 6.4A). Normal F9 fibroblasts co-expressing both apoptin and shRNA directed against B56γ mRNA manifested a clear level of phosphorylated apoptin in comparison to the cells transfected with apoptin and the RNAi control vector (Figure 6.4C). Our data thus indicate that inhibition of the PP2A B56γ subunit is a crucial and sufficient step for apoptin phosphorylation.

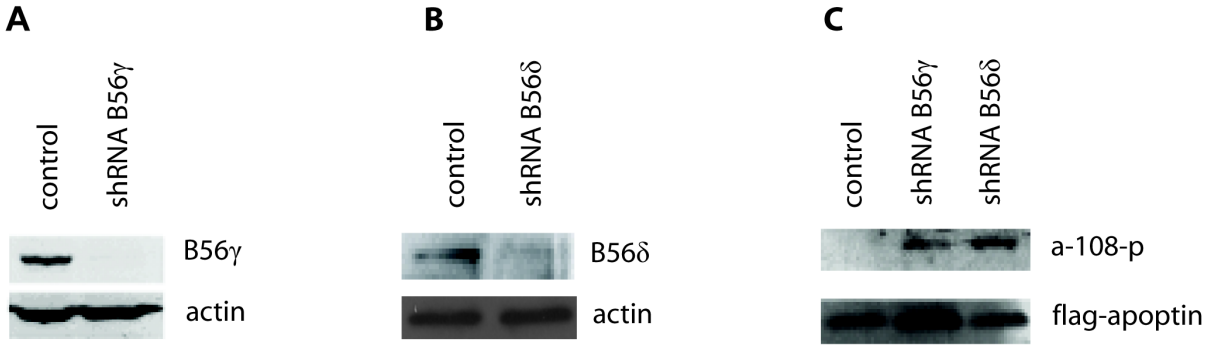

Figure 6.4 Knock-down of PP2A B56γ and B56δ subunits triggers apoptin phosphorylation in normal cells. **A.** Down-regulation of PP2A B56γ subunit by shRNA. HeLa cells were co-transfected with pCEP-4HA-B56γ expressing 4HA-tagged B56γ, and either shB56γ or control pSuper vector and lysed 48h post-transfection, followed by western blotting analysis with the indicated antibodies. **B.** Down-regulation of PP2A B56δ subunit by shRNA. F9 cells were transfected with pSuper vector encoding shRNA directed against PP2A B56δ or pSuper vector control; 24h post-transfection, cell lysates were prepared and subsequently analyzed by Western blot. **C.** F9 cells were co-transfected with Flag-apoptin and either pSuper vector encoding shRNA directed against PP2A B56γ, δ or control; 24-48h post-transfection, cell lysates were prepared and subsequently analyzed by Western blot.

Over-expression of PKA-interacting protein BCA3 stimulates apoptin activity in tumor cells

Analogous to the enhancing effect of ST on apoptin phosphorylation in normal cells, we observed an enhancement of apoptin phosphorylation in tumor cells by BCA3. BCA3 was identified as an apoptin-interacting protein by means of a yeast two-hybrid assay, and interacts with apoptin in a human cellular background (Figure 6.5A). Co-expression of BCA3 and Flag-apoptin in human Saos-2 tumor cells resulted in a significant increase in the apoptosis activity of apoptin (Figure 6.5B). In fact, as early as 6 hours after transfection, phosphorylated apoptin could readily be detected in Saos-2 cells expressing both apoptin and BCA3, whereas in cells expressing apoptin alone (control), apoptin phosphorylation was not yet visible at this early time-point (Figure 6.5C).

As BCA3 (9) has been shown to interact with the catalytic subunit of PKA (Sastri, et al., 2005), the involvement of PKA in apoptin phosphorylation was investigated. Treatment of Saos-2 cells with H89, a known PKA inhibitor (Lochner and Moolman, 2006), enhanced apoptin phosphorylation. In

contrast, addition of the PKA activator cAMP (Bulun and Simpson, 2008) diminished apoptin phosphorylation (Figure 6.5D). These results suggest that interference with PKA activity favors activation of apoptin. Taking into account the fact that PP2A has been shown to be a target of PKA (Ahn, et al., 2007), this provides another link pointing to the involvement of PP2A in the activation of apoptin.

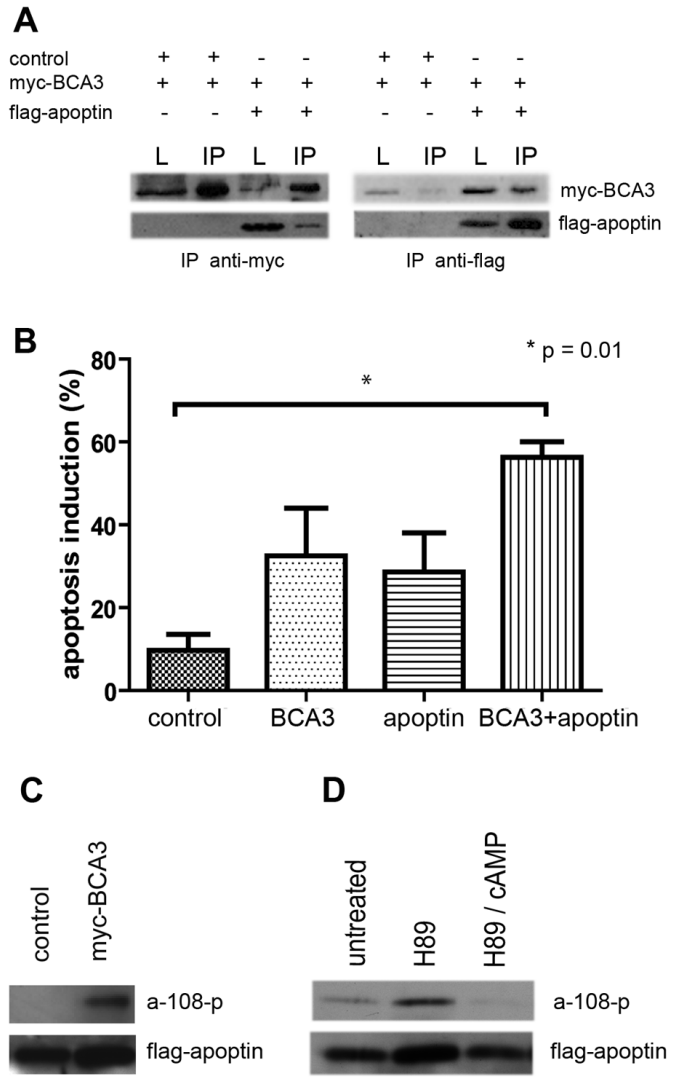

Figure 6.5 BCA3 interacts with apoptin and stimulates its activity. **A.** Apoptin interacts with BCA3 in a cellular background. Normal human foreskin fibroblasts were transfected with plasmids encoding myc-tagged BCA3 and Flag-tagged apoptin, or control plasmid in the indicated combinations. Total lysates (L) or protein complexes immunoprecipitated (IP) with antibody against the myc- (left panel) or Flag-tags (right panel) were separated by SDS-PAGE and analyzed by Western blot. **B.** Expression of myc-BCA3 together with Flag-apoptin results in increased induction of apoptosis. Human Saos-2 tumor cells were transfected with plasmids encoding Flag-tagged apoptin and myc-tagged BCA3, or vector control in the indicated combinations and grown on glass coverslips. Forty-eight hours post-transfection, slides were fixed and stained with appropriate antibodies for immunofluorescence analysis (right panel). pMaxGFP (Amaxa) was used as a negative control for apoptosis. Data are representative of 3 independent experiments, in which at least 100 cells were scored. **C.** Co-expression of myc-BCA3 enhances apoptin phosphorylation. Saos-2 cells transfected with BCA3 and apoptin, or apoptin alone, were lysed 6 hours post-transfection and analyzed for apoptin phoshphorylation by Western blot analysis. **D.** Inhibition of PKA results in increased apoptin phosphorylation. Saos-2 cells were transfected with Flag-tagged apoptin, and treated with the PKA inhibitor H89 (1h, 10μM) or activator cAMP (30 minutes, 10μM H89, followed by 30 minutes 1mM cAMP) 24hs post-transfection. Cells were then lysed and their contents analyzed by Western blot using indicated antibodies.

Down-regulation of PP2A B56δ subunit activates apoptin phosphorylation in normal cells

Ahn et al. reported that the PP2A B56δ subunit is phosphorylated by PKA, thereby increasing the overall activity of PP2A. The activity of a B56δ mutant that cannot be phosphorylated by PKA is significantly less than its wild-type counterpart (Ahn, et al., 2007). Therefore, one can assume that inhibition of PKA results in a diminished functional PP2A B56δ subunit, and hence aberrant PP2A activity.

We examined whether inhibition of the expression of the PP2A B56δ subunit in human F9 cells positively affected the phosphorylation of apoptin. Down-regulation of B56δ protein expression through RNAi was confirmed in normal F9 fibroblasts (Figure 6.4B). Co-expression of shRNA targeting B56δ mRNA and apoptin in normal F9 cells clearly resulted in the activation of apoptin phosphorylation, as compared to F9 cells transfected with apoptin and the RNAi control vector (Figure 6.4C).

Our results imply that PP2A complexes containing the regulatory subunits B56 γ and δ are essential for maintaining a normal cell environment, as the loss of either one of these subunits results in the activation of apoptin, which is typical for cancer cells.

Discussion

Activation of tumor-selective apoptosis-inducing proteins is an intriguing phenomenon, and revealing the molecular switch behind this process could allow important insights for developing anticancer therapies. Apoptin was the first protein known to harbor apoptosis activity selectively in transformed human cells (Danen-van Oorschot, et al., 1997). However, the molecular mechanisms underlying apoptin's tumor-selective activity are largely unknown (Grimm and Noteborn, 2010). Here, our studies reveal that apoptin becomes activated by inhibition of normal PP2A function. Two independent

lines of research pointed to a fundamental role of PP2A in the regulation of apoptin activity.

In one study, we examined the effect of specific domains within the transforming SV40 proteins LT136 and ST on the activation of apoptin. The C-terminal ST PP2A-binding transformation domain (Arroyo and Hahn, 2005; Pipas, 2009), when targeted to the nucleus of normal cells, turned out to be crucial for the tumor-characteristic activation of apoptin. We showed that a C103S mutation within the ST PP2A binding site (Chen, et al., 2004) abrogated the phosphorylation of apoptin induced by NLS-ST in normal human fibroblasts. RNA interference studies confirmed that inactivation of the B56γ protein promotes phosphorylation of apoptin in human fibroblasts.

A second line of research indicated that interference with the PP2A B56δ domain also led to the activation of apoptin. Over-expression of the apoptin- and PKA-interacting partner BCA3, or inhibition of PKA by treatment with H89 both resulted in enhanced phosphorylation and apoptosis activity of apoptin in human cancer cells. PKA is known to phosphorylate the B56δ subunit of PP2A leading to increased PP2A activity (Ahn, et al., 2007). RNA interference studies showed that inhibition of the synthesis of the PKA target PP2A-B56δ indeed activated apoptin phosphorylation in normal human cells.

Our studies, carried out from two different angles of research, reveal that apoptin senses PP2A inactivation during malignant cellular transformation. Interestingly, the delta and gamma subunits are the only nuclear PP2A B56 subunits (Chen, et al., 2004; McCright, et al., 1996), and nuclear localization is important for both LT/ST-induced cell transformation and apoptin-induced tumor-selective apoptosis. PP2A complexes containing B56δ domains prevent entry of cells into mitosis upon DNA damage (Virshup and Shenolikar, 2009), averting genetic instability that might contribute to cell transformation. B56γ mediates dephosphorylation and stabilization of the tumor suppressor protein p53 upon DNA damage, inhibiting cellular

proliferation and transformation (Li, et al., 2007). Studies have shown that derailment of B56γ results in aberrancies in functioning of e.g. cell cycle and tumor suppressor proteins, resulting in cell transformation (Chen, et al., 2004; Sablina and Hahn, 2008).

PP2A has also been shown to play a role in the activation of other tumor-selective apoptosis-inducing proteins. Besides apoptin, also the adenovirus E4orf4 protein has been shown to selectively induce apoptosis in human cancer cells (Branton, et al., 2001; Kleinberger, 2000). Direct interaction of E4orf4 with PP2A regulatory B domains is essential for the tumor-selective apoptosis activity of E4orf4 (Strichman, et al., 2000). Interaction of E4orf4 with the PP2A B55 subunit results in down-regulation of the expression of the Myc oncogene (Ben-Israel, et al., 2008). In addition to SV40 ST, other viral transforming proteins also interact with PP2A, showing its relevance in cellular transformation (Zhao and Elder, 2005). These findings corroborate earlier reports that aberrant PP2A functioning activates transforming processes, which then elicit the activation of tumor-selective apoptosis-inducing proteins such as apoptin or E4orf4.

Further steps within the development of tumorigenic cells seem at least not critical for apoptin's tumor-selective apoptosis characteristics. These conclusions are in accordance with the observations by others and ourselves that apoptin is able to induce apoptosis in a very broad panel of tumor types (Backendorf, et al., 2008; Grimm and Noteborn, 2010; Maddika, et al., 2006; Tavassoli, et al., 2005). If one assumes that tumor cells arise by a wide variety of mechanisms, all the while sharing a limited number of key characteristics, then apoptin simply needs to recognize one (or a subset) of these characteristics.

We have shown that inactivation of the nuclear PP2A B56 γ and/or δ subunits is sufficient to trigger apoptin's tumor-selective apoptosis activity. PP2A provides a central phosphatase activity affecting many cellular signaling pathways, and derailment of PP2A activity seems to be one of the

fundamental events occurring during oncogenic transformation. This implies that apoptin might be applied for the treatment of tumors arising from a wide range of origins.

Acknowledgements

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References

Ahn JH, McAvoy T, Rakhilin SV, Nishi A, Greengard P, Nairn AC (2007) Protein kinase A activates protein phosphatase 2A by phosphorylation of the B56δ subunit. *Proc Natl Acad Sci U S A* 104, 2979-2984.

Alvisi G, Poon IK and Jans DA (2006) Tumor-specific nuclear targeting: promises for anti-cancer therapy. *Drug Resist Updat* 9, 40-50.

Arnold HK and Sears RC (2006) Protein phosphatase 2A regulatory subunit B56alpha associates with c-myc and negatively regulates c-myc accumulation. *Mol Cell Biol* 26, 2832-2844.

Arroyo JD and Hahn WC (2005) Involvement of PP2A in viral and cellular transformation. *Oncogene* 24, 7746-7755.

Backendorf C, Visser A, De Boer A, Zimmerman R, Visser M, Voskamp P et al. (2008) Apoptin: Therapeutic potential of an early sensor of carcinogenic transformation. *Annu Rev Pharmacol Toxicol* 48, 143-169.

Ben-Israel H, Sharf R, Rechavi G and Kleinberger T (2008) Adenovirus E4orf4 protein down-regulates Myc expression through interaction with PP2A-B55 subunit. *J Virol* 82, 9381-9388.

Boutros M and Ahringer J (2008) The art and design of genetic screens: RNA interference. *Nat Rev Genet* 9, 554-566.

Branton PE and Roopchand DE (2001) The role of adenovirus E4orf4 protein in viral replication and cell killing. *Oncogene* 20, 7855-7865.

Bruno P, Brinkmann C, Boulanger M, Flinterman M, Klanrit P, Landry M et al. (2009) Family at last: highlights of the first international meeting on proteins killing tumour cells. *Cell Death and Differentiation* 16, 184-186.

Bulun SE and Simpson ER (2008) Aromatase expression in women's cancers. *Adv Exp Med Biol* 630, 112-132.

Chen W, Possemato R, Campbell KT, Plattner CA, Pallas DC, Hahn WC (2004) Identification of specific PP2A complexes involved in human cell transformation. *Cancer Cell* 5, 127-136.

Chen Y, Xu Y, Bao Q, Xing Y, Li Z, Lin Z et al. (2007) Structural and biochemical insights into the regulation of protein phosphatase 2A by small t antigen of SV40. *Nat Struc Mol Biol* 6, 527-534.

Cho US, Morrone S, Sablina AA, Arroyo JD, Hahn WC, Xu W (2007) Structural basis of PP2A inhibition by small t antigen. *PLoS Biol* 5, e202.

Danen-Van Oorschot AAAM, Fischer DF, Grimbergen JM, Klein B, Zhuang Y-H, Falkenburg JHF et al. (1997) Apoptin induces apoptosis in human transformed and malignant cells but not in normal cells. *Proc Natl Acad Sci U S A* 94, 5843-5847.

Danen-Van Oorschot AAAM, Voskamp P, Seelen MCMJ, Miltenburg MHAM, Bolk MW, Tait SW et al. (2004) Human death effector domain-associated factor interacts with the viral apoptosis agonist Apoptin and exerts tumor-preferential cell killing. *Cell Death Differ* 11, 564-573.

Eichhorn PJ and Creyghton MP, Bernards R (2009) Protein phosphatase 2A regulating subunits and cancer. *Biochem Biophys Acta* 2795,1-15.

Fahrbach KM, Katzman RB and Rundell K (2008) Role of SV40 ST antigen in the persistent infection of mesothelial cells. *Virology* 379, 255-263.

Gjoerup O, Zaveri D, and Roberts TM (2001) Induction of p53-independent apoptosis by simian 40 small t antigen. *J Virol* 75, 9142-9155.

Grimm S and Noteborn M (2010) Anti-cancer genes: inducers of tumour-specific cell death signalling. *Trends in Molecular Medicine* 16, 88-96.

Kitching R, Li H, Wong MJ, Kanaganayakam S, Kahn H, and Seth A (2003) Characterization of a novel human breast cancer associated gene (BCA3) encoding an alternatively spliced proline-rich protein. *Biochim Biophys Acta* 1625, 116-121.

Kleinberger T (2000) Induction of apoptosis by adenovirus E4orf4 protein. *Apoptosis* 5, 211-215.

Li H, Cai X, Shouse GP, Piluso LG and Liu X (2007) A specific PP2A regulatory subunit, B56γ, mediates DNA damage-induced dephosphorylation of p53 at Thr55. *EMBO J* 26, 402–411.

Lochner A and Moolman JA (2006) The many faces of H89: a review. *Cardiovasc Drug Rev* 24, 261-274.

Maddika S, Mendosa FJ, Hauff K, Zamzow CR, Paranjothy T, Los M (2006) Cancer-selective therapy of the future: apoptin and its mechanism of action. *Cancer Biol Ther* 5, 10-19.

McCright B, Rivers AM, Audin S and Virshup DM (1996) The B56 family of protein phosphatise 2A (PP2A) regulatory subunits encodes differentiation-induced phosphoproteins that target PP2A to both nucleus and cytoplasm. *J Biol Chem* 271, 22081-22089.

Naviglio S, Caraglia M, Abbruzzese A, Chiosi E, Di Gesto D, Marra M et al. (2009) Protein kinase A as a biological target in cancer therapy. *Expert Opin Ther Targets* 13, 83-92.

Noteborn MHM, De Boer GF, Van Roozelaar DJ, Karreman C, Kranenburg O, Vos JG et al. (1991) Characterization of cloned chicken anemia virus DNA that contains all elements for the infectious replication cycle. *J Virol* 65, 3131-3139.

Pipas J (2009) SV40: Cell transformation and tumorigenesis. *Virology* 384, 294-303.

Rohn JL and Noteborn MHM. (2004) The viral death effector apoptin reveals tumor-specific processes. *Apoptosis* 9, 315-322.

Rohn JL, Zhang Y-H, Aalbers RIJM, Otto N, Den Hertog J, Henriquez NV, et al. (2002) A tumor-specific kinase activity regulates the viral death protein apoptin. *J Biol Chem* 277, 50820-50827.

Sablina AA and Hahn WC (2008) SV40 small T antigen and PP2A phosphatase in cell transformation. *Cancer Metastasis Rev* 27,137-146.

Sablina AA, Hector M, Colpaert N and Hahn WC (2010) Identification of PP2A complexes and pathways involved in cell transformation. *Cancer Res* 70, 10474-10484.

Sastri M, Barraclough DM, Carmichael PT and Taylor SS (2005) A-kinase-interacting protein localizes protein kinase A in the nucleus. *Proc Natl Acad Sci U S A* 102, 349-354.

Schmidt TGM and Skerra A (2007) The Strep-tag system for one-strep purification and high-affinity detection or capturing of proteins. *Nature Protocols* 2, 1528-1535.

Srinivasan A, McClellan AJ, Vartikar J, Marks I, Cantalupo P, Li Y et al. (1997) The amino-terminal transforming region of simian virus 40 large T and small t antigens functions as a J domain. *Mol Cell Biol* 17,4761-4773.

Strichman R, Sharf R and Kleinberger T (2000) Adenovirus E4orf4 protein interacts with both Balpha and B' subunits of protein phosphatase 2A but E4orf4-induced apoptosis is mediated only by its interaction with Balpha. *Oncogene* 19, 3757-3763.

Tavassoli M, Guelen L, Luxon BA and Gaken J (2005) Apoptin: specific killer of tumor cells? *Apoptosis* 10, 717-724.

Usui H, Inoue R, Tanabe O, Nishito Y, Shimizu M, Hayashi H et al. (1998) Activation of protein phosphatase 2A by cAMP-dependent protein kinase-catalyzed phosphorylation of the 74-kDa B" (delta) regulatory subunit in vitro and identification of the phosphorylation sites. *FEBS Lett* 430, 312-316.

Van Kanegan MJ and Strack S (2009) The protein phosphatase 2A regulatory subunits B'beta and B'delta mediate sustained TrkA neurotrophin receptor autophosphorylation and neuronal differentiation. *Mol Cell Biol* 29, 662-674

Virshup DM and Shenolikar S (2009) From promiscuity to precision: protein phosphatases get a makeover. *Molecular Cell* 33, 537-545.

Wenner CE (2010) Cell signaling and cancer - possible targets for therapy. *J Cell Physiol* 223, 299-308.

Westermarck J and Hahn W (2008) Multiple pathways regulated by the tumor suppressor PP2A in transformation. *Trends Mol Med* 14,152-60.

Yamashita K, Ikenaka Y, Kakutani T, Kawaharada H, and Watanabe K (1990) Comparison of human lymphotoxin gene expression in CHO cells directed by genomic DNA or cDNA sequences. *Agric Biol Chem* 54, 2801-2809.

Zhang YH, Kooistra K, Pietersen AM, Rohn JL, and Noteborn MH (2004) Activation of the tumor-specific death effector apoptin and its kinase by an N-terminal determinant of simian virus 40 large T antigen. *J Virol* 78, 9965-9976.

Zhao RY and Elder RT (2005) Viral infections and cell cycle G2/M regulation. *Cell Res* 115, 143-149.

Chapter 7

Discussion, outlook and conclusions

Mechanisms behind the tumor-specific apoptosis inducing protein apoptin: clues from apoptin-interacting proteins

Abstract

With the discovery of apoptin's unique capability to induce tumor-specific apoptosis, came the promise of the development of a potentially powerful, yet safe and effective, novel concept in anti-cancer therapy. Efforts to uncover the mechanisms underlying the differential behavior of apoptin in normal and transformed cells identified a number of interacting proteins – including Dedaf in tumor cells, and Hippi in normal cells – while a central dogma emerged: in tumor cells, apoptin was postulated to be phosphorylated, move to the nucleus and induce apoptosis, whereas in normal cells, according to the same dogma, apoptin would not be phosphorylated, remained in the cytoplasm and would eventually be eliminated from the cell. Recent research presented in this thesis, has provided important new clues into the workings of this enigmatic protein. This chapter discusses these results against the backdrop of nearly two decades of apoptin research, highlighting the roles of new, key interacting partners of apoptin in the human cell. Novel insights into the pathways underlying apoptin cellular function are discussed, as well as how these could be used to improve cancer therapy.

The discovery of apoptin and its establishment as a Protein Killing Tumor Cells

As introduced in **chapter 2**, apoptin is a small, proline-rich, protein encoded by the Chicken Anemia Virus (CAV; figure 7.1). Infection of young chicks with CAV causes anemia and immunodeficiency due to apoptosis of bone marrow, splenic and thymus cells, and apoptin was identified as the agent responsible for the induction of apoptosis (Jeurissen, et al., 1992; Noteborn, et al., 1994; Noteborn and Koch, 1995). Upon over-expression in human tumor cells, apoptin was shown to induce apoptosis in these cells as well (Zhuang, et al., 1995, 1995b), and even more excitingly, it did not do so when overexpressed in normal human cells (Danen-van Oorschot, et al., 1997; Noteborn, et al., 1998).

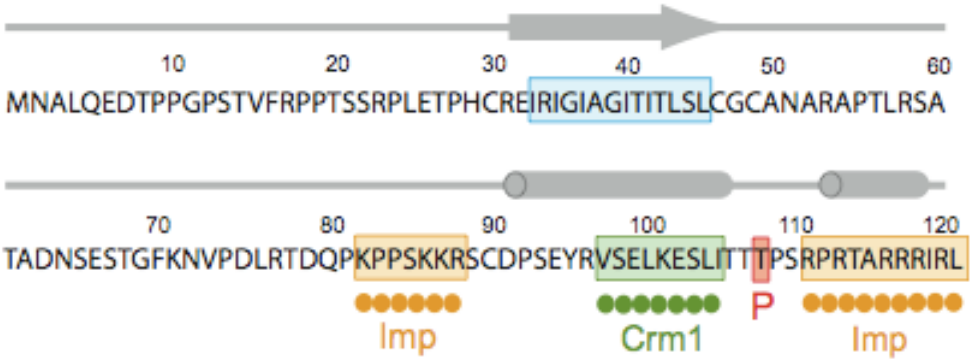

Figure 7.1 Schematic representation of the apoptin protein. A putative β-strand and two α-helices, as predicted by the PSIPRED software, are indicated. The N-terminal (iso)leucine-rich, multimerization and protein-protein interaction domain is indicated in blue, the bi-partite NLS is indicated in yellow, surrounding the NES in green, and the T108 phosphorylation site in red. Adapted from Backendorf, et al., 2008.

Further comparison of the differential behavior of apoptin in normal and cancer cells revealed that apoptin had a cytoplasmic localization in the former, versus a nuclear localization in the latter (Danen-van Oorschot, et al., 1997). A bipartite nuclear localization signal (Poon, et al., 2005; Danen-van Oorschot, et al., 2003) surrounding a nuclear export signal was identified between amino acids 80-121 (Poon, et al., 2005b). Located precisely in this region, a threonine amino acid at position 108 was identified

as the target of a yet unknown kinase activity, which appeared to be present in tumor cells but lacking in normal cells (Rohn, et al., 2002). Hence, it was hypothesized that apoptin was able to shuttle between the nucleus and cytoplasm, accumulating in the nucleus of tumor cells upon phoshorylation of T108, which would block the nuclear export signal (NES) while leaving the nuclear location signal (NLS) exposed. In normal cells, it would remain in the cytoplasm as a result of the exposed NES, where it would eventually be degraded and eliminated from the cell (Zhang, et al., 2003). Poon et al. (2005b) postulated that the phosphorylation of apoptin would most likely take place in the (tumor) cell nucleus. This was, however, challenged by the fact that phosphorylated apoptin could also be found in the cytoplasm of tumor cells (Zhang, et al., 2004; R. Zimmerman, unpublished results), consistent with recent findings in our laboratory that apoptin kinase activity can be found in both the cytoplasm and nucleus of various cancer cell lines (Lanz, et al., 2012).

Immunoelectron microscopy determined that, within the nucleus, apoptin colocalized with heterochromatin and nucleoli, and apoptin was shown to bind DNA *in vitro* (Leliveld, et al., 2003, 2004). Intriguingly, however, experiments using actinomycin D, an RNA synthesis inhibitor, and two protein synthesis inhibitors (emetine and puromycin), demonstrated that apoptin's cell-killing effects did not require *de novo* transcription or translation (Danen-van Oorschot, et al., 2003). Biochemical analysis further showed that apoptin functioned as a multimeric protein, with each multimer consisting of approximately 20-40 monomeric subunits (Leliveld, et al., 2003). An N-terminal amphiphatic helix (amino acids 29-49) was identified as the multimerization domain; this multimerization domain was also postulated to be the interacting domain with other cellular proteins.

Apoptin and its interacting proteins

In an attempt to elucidate the molecular mechanisms involved in a) apoptin's discrimination between normal and human cells, and b) the subsequent induction of apoptosis, human cellular proteins interacting with apoptin

were sought and identified. Below, we recapitulate the most important interactions found in literature, as well as the novel interacting proteins presented in this thesis (see Table 7-1 at the end of this chapter).

PI3-K and Akt

Class I phosphatidylinositol 3-kinases (PI3-Ks) play a central role in various regulatory processes, such as cell growth, survival and differentiation. As discussed in **chapter 2**, they are also involved in autophagic cell death, and can function both to limit and promote tumor development. The downstream effectors of PI3K include the Akt kinase (Marte and Downward, 1997; Vanhaesebroeck and Waterfield, 1999), which modulates the function of numerous substrates related to the regulation of cell proliferation, such as cyclin-dependent kinase inhibitors, p21Cip1/Waf1 (Li, et al., 2002), p27kip1, (Fruman, et al., 1998; Song, et al., 2005) and Nur77 (Pekarsky, et al., 2001). Another important function of activated PI3-K/Akt in cells is maintaining cell survival by inhibition of apoptosis, e.g. through Akt phosphorylation of Bad (Coffer, et al., 1998).

Apoptin was shown to interact with the p85 regulatory subunit of PI3-K in both normal and cancer cells, leading to constitutive activation of PI3-K (Maddika, et al., 2008). Inhibition of PI3-K activation either by chemical inhibitors or by genetic approaches severely impaired cell death induced by apoptin. Downstream of PI3-K, Akt was activated and translocated to the nucleus together with apoptin. Direct interaction between apoptin and Akt was documented, and co-expression of nuclear Akt significantly potentiated cell death induced by apoptin (Maddika, et al., 2007). Accordingly, Nur77, one of the targets of activated Akt, was previously shown to translocate from the nucleus to mitochondria during apoptin-induced cell death (Maddika, et al., 2005).

Hippi

In **chapter 2**, we briefly alluded to the interaction between apoptin and Hippi. Hippi is the protein interactor and apoptosis co-mediator of

Huntingtin interacting protein 1 (Hip1). Diverse cellular functions have been described for Hippi (also known as estrogen-related receptor beta like 1 or intraflagellar transport 57 homolog). In Huntington's disease, the Huntingtin (Htt) protein undergoes polyglutamine repeat expansion and loses its ability to interact with the endocytic protein Hip1. This allows Hip1/Hippi complexes to form and in turn trigger caspase-8-mediated apoptosis (Gervais, et al., 2002). Moreover, Hippi was found to be a transcriptional regulator of caspase expression: exogenous expression of HIPPI increases expression of caspases-1, -8 and -10 in HeLa and Neuro2A cells and induces apoptosis (Majumder, et al., 2007; 2007b). In addition to this possible caspase-dependent mechanism for pathological cell death in Huntington's disease (Ferrier, 2002), Hippi was also reported to activate the mitochondrial (intrinsic) apoptotic pathway in neuronal cells through Bid cleavage (Majumder, et al., 2006), and to bind other apoptosis-related proteins, including apoptin, and the apoptin-interacting protein Rybp/DEDAF (Stanton, et al., 2007; see below).

Mapping studies indicate that Hippi binds within the self-multimerization domain of apoptin, and apoptin binds to the C-terminal half of Hippi, including its death effector domain-like motif (Cheng, et al., 2003). Subcellular localization studies showed that Hippi and apoptin perfectly colocalized in the cytoplasm of normal human HEL cells, whereas in cancerous HeLa cells most apoptin and Hippi were located separately in the nucleus and cytoplasm, respectively. These results suggested that the interaction with Hippi might play a role in the suppression of apoptin-induced apoptosis in normal cells. However, over-expression of Hippi in HeLa cells did not prevent apoptin nuclear accumulation, nor did it inhibit its apoptotic activity, indicating that there is still another factor involved in determining the cytoplasmic localization of apoptin and inhibition of apoptin-induced apoptosis in normal cells (Cheng, et al., 2003).

*FAM96B (**Chapter 4**)*

Family with sequence similarity 96, member B (FAM96B) is a highly conserved protein, whose function was until recently unknown. Proteins sharing its conserved domain DUF59 serve a variety of roles, including phenylacetic acid degradation, iron-sulfur cluster biosynthesis, nucleotide-binding and chromosome partitioning, and calcium-dependent protein phosphorylation (Finn, et al., 2010).

We found that FAM96B interacts with apoptin in both normal and tumor cells (**chapter 4**). Interestingly, this function was independent of apoptin phosphorylation. Proteomic analysis suggests FAM96B is involved in the establishment of sister chromatid cohesion and subsequent chromosome segregation (**chapter 4**; Ben Arroya, et al., 2008; Ito, et al., 2010). FAM96B associates with various proteins involved in DNA replication and repair, as well as, cytoskeletal components, effectively linking the DNA damage response to sister chromatid cohesion and segregation, and cell cycle regulation. Preliminary results indicate FAM96B is down-regulated in tumor cells (R. Zimmerman, J. Tian and C. Backendorf, unpublished results). Furthermore, studies in yeast (Ben-Aroya, et al., 2008) and preliminary observations in zebrafish (R. Zimmerman, unpublished results) indicate that FAM96B is an essential gene required for normal embryonic development. This implies that ectopic expression of FAM96B reimposes a cellular control mechanism (most likely regarding its function in linking the DNA damage response to control of the mitotic spindle), which is recognized by apoptin: in normal cells, this prevents apoptin-induced apoptosis, whereas in tumor cells, where this control mechanism is lacking, apoptin-induced apoptosis is triggered.

*RYBP/DEDAF and other nucleolar proteins (**chapter 5**)*

One of the proteins found to associate with FAM96B is Rybp/DEDAF (**chapter 4**). Coincidentally, the same protein was also found to interact with both Hippi (Stanton, et al., 2007) and apoptin (Danen-van Oorschot, et al., 2004).

Rybp (RING1 and YY1 binding protein) belongs to the Rybp/Yaf2 family of small, basic, Npl4 zinc finger (NZF)-containing proteins that have been highly conserved throughout evolution. Initially, Rybp was characterized as an interacting partner for proteins involved in transcriptional regulation, such as YY1 and members of the E2F family, as well as with transcriptional co-repressors such as Polycomb group proteins (Bejarano, et al., 2005; Garcia, et al., 1999; Sawa, et al., 2002; Schlisio, et al., 2002; Trimarchi, et al., 2001). Importantly, Rybp is involved in the p53 response to DNA damage, by interacting with MDM2 and decreasing MDM2-mediated p53 ubiquitination, leading to stabilization of p53 and cell-cycle arrest (Chen, et al., 2009).

Beyond this, Rybp has been implicated in the promotion of apoptosis. Rybp binds heterotypically to several DED-containing apoptotic mediators, and accordingly is also known as DEDAF, for death effector domain associated factor (Zheng, et al., 2001). Homophilic interactions of death effector domains (DEDs) are crucial for the signaling pathways of death receptor-mediated apoptosis. Rybp/DEDAF interacts with FADD, procaspase-8, and procaspase-10 in the cytosol, as well as with the DED-containing DNA-binding protein (DEDD) in the nucleus. It can enhance both death receptor- and caspase-10 DED-mediated apoptosis in lymphoma cell lines (Zheng, et al., 2001). At the cell membrane, it augmented the formation of CD95-FADD-caspase-8 complexes and enhanced death receptor- as well as DED-mediated apoptosis. In the nucleus, Rybp/DEDAF caused the DEDD protein to relocalize from subnuclear structures to a diffuse distribution in the nucleoplasm.

As mentioned before, Rybp/DEDAF also interacts with apoptin and has been suggested, like apoptin, to induce apoptosis preferentially in transformed cell lines (Danen-van Oorschot, et al., 2004; Novak and Phillips, 2008). Accordingly, expression of Rybp/DEDAF is decreased in human cancer tissues compared with adjacent normal tissues. Rybp/DEDAF is predominantly nuclear and partially co-localizes with apoptin in intact and

apoptotic tumor cells, but not in normal cells, where it is nuclear while apoptin remains cytoplasmic.

In **chapter 5**, apoptin was demonstrated to interact with chromatin, and possibly nucleolar (R-)chromatin. Indeed, proteins co-purifying with chromatin-associated apoptin complexes included the nucleolar proteins Cdc5L, nucleophosmin and nucleolin, as well as components of the mitotic spindle and the PP2A inhibiting, cell cycle regulatory protein SET1. Like DEDAF, both Cdc5L and nucleophosmin play important roles in the response to DNA damage and maintenance of genomic stability; in addition, Cdc5L and SET1 are involved in the progression of both S-phase and the G2/M transition. Nucleophosmin is also involved in cellular growth through nucleocytoplasmic export of ribosomes, and centrosome duplication and mitotic spindle formation, with nucleolin sharing similar functions. Together with the identification of proteins involved in ribosome biogenesis and RNA metabolism, the data suggest that apoptin orchestrates its apoptotic effects from within the nucleolus, where it senses DNA damage and shuts down the cellular protein factory.

Rsf-1

One of the apoptin-interacting proteins found by yeast-two-hybrid assay is Rsf-1. We found that Rsf-1 interacted and colocalized with apoptin in the nucleus of human tumor cells (R. Zimmerman and A. Danen-van Oorschot, unpublished results). RSF is a heterodimer of the PHD-finger containing protein Rsf-1 and the SNF2H ATPase belonging to the ISWI family, and a unique chromatin remodeling factor that can assemble regularly spaced nucleosomal arrays without the aid of additional histone chaperones (Loyola, et al., 2001; 2003). Such nucleosome remodeling is essential for transcriptional activation or repression (Vignali, et al., 2000), DNA replication (Flanagan and Peterson, 1999), and cell cycle progression (Cosma, et al., 1999). Rsf-1 was found to play a role in silent chromatin formation by promoting histone H2Av replacement. H2Av is the *Drosophila* variant of mammalian H2AZ, a histone H2A variant that is essential for

establishing proper chromatin structure in many organisms (Raisner and Madhani, 2006; Greaves, et al., 2007; Rangasamy, et al., 2004), and is involved in the formation of constitutive, as well as, facultative heterochromatin (Greaves, et al., 2006; Sarcinella, et al., 2007). *Drosophila* H2Av mutants exhibit reduced H3 lysine 9 (H3K9) methylation and HP1 binding (Swaminathan, et al., 2005), both of which are required to recruit Suv420h1/2 for the establishment of heterochromatin (Schotta, et al., 2004). In *Drosophila melanogaster*, loss of RSF function reduced the levels of the histone variant H2Av and histone H3-K9 methylation, and suppressed silencing of transcription in a euchromatic region near the centromeric heterochromatin (Hanai, et al., 2008).

RSF was also found to interact with CENP-A chromatin, a histone H3 variant that is crucial to the formation of centromeric chromatin (Perpelescu, et al., 2009). Rsf-1 depletion induced loss of centromeric CENP-A, and purified RSF complex reconstituted and spaced CENP-A nucleosomes *in vitro*, further implicating the involvement of Rsf-1 in this process.

The sequence of the gene for Rsf-1 is located on chromosome 11q13, and includes that for HBXAP, which was postulated to be involved in the transcriptional regulation of the hepatitis B virus (Shamay, et al., 2002). HBXAP actually contains a 252-amino-acid truncation of the amino terminus of Rsf-1, which lacks the ISWI-interaction domain, and therefore is not functional within the RSF complex. Amplification at the 11q13 locus is commonly observed in breast, ovarian, head and neck, oral, and esophageal cancer (Mao, et al., 2006; Schwab, 1998; Shih Ie, et al., 2005; Nakayama, et al., 2007). Patients with Rsf-1 amplification or over-expression had a significantly shorter overall survival than those without (Shih Ie, et al., 2005). *In vitro,* over-expression of the Rsf-1 gene stimulated cell proliferation and transformation of nonneoplastic cells by conferring serum-independent and anchorage-independent growth. Furthermore, Rsf-1 gene knockdown inhibited cell growth in OVCAR3 cells, which harbor Rsf-1 amplification (Shih Ie, et al., 2005). The association of Rsf-1 amplification cq over-

expression with worse survival in (ovarian) cancer patients, combined with the stimulatory effect on cell proliferation *in vitro*, is similar to the properties of known oncogenes, including HER2/neu in breast cancer (Borg, et al., 1990; Tsuda, et al., 1989) and N-myc in neuroblastoma (Rubie, et al., 1997). This suggests that Rsf-1 itself has oncogenic potential; consistent with such a function, Rsf-1 was found to be up-regulated in paclitaxel-resistant ovarian cancer cell lines, and *Rsf-1* gene knockdown sensitized tumor cells to paclitaxel (Choi, et al., 2009). Down-regulation of hSNF2H or disruption of hSNF2H and Rsf-1 interaction also enhanced paclitaxel sensitivity in tumor cells with Rsf-1 up-regulation. On the other hand, ectopic expression of Rsf-1 significantly enhanced paclitaxel resistance in ovarian cancer cells.

Preliminary observations showed that co-expression of Rsf-1 together with apoptin stimulates apoptin-induced apoptosis (R. Zimmerman, unpublished results), corroborating both the oncogenic potential of Rsf-1 and the preference of apoptin for a transformed environment.

Suv420h1

Suppressor of variegation 4-20 homolog 1, or Suv420h1, is a SET-domain containing protein, which was found to interact with apoptin in a yeast two-hybrid assay (A. Danen-van Oorschot, unpublished results). SET domains are found in histone methyl transferases (HMTs) (Qian and Zhou, 2006), and also serve as interaction domains with dual-specificity protein phosphatases (dsPTPases) (Cui, et al., 1998). Intriguingly, CAV VP2 has been shown to encode such a dsPTPase (Peters, et al., 2002), and SET domain-dsPTPase interactions appear to be critically important for regulating the growth properties of lymphoid progenitors (De Vivo, et al., 1998), which are among the preferentially targeted cell types during CAV infection (Adair, 2000).

Drosophila Suv4-20 is a mixed product specificity methyltransferase that dimethylates approximately 90% and trimethylates less than 5% of total H4 at lysine 20 in S2 cells (Yang, et al., 2008). Similar to the Drosophila enzyme, human Suv4-20h1/h2 enzymes generate di- and trimethyl H4K20

(Schotta, et al., 2004; 2008; Yang, et al., 2008). Dimethyl H4K20 has been shown to recruit the p53 binding protein 53BP1 to DNA damage foci in the fission yeast *S. pombe*, and is essential for the proper execution of the DNA damage response (Sanders, et al., 2004; Greeson, et al., 2008). Depletion of Suv4-20h1/2 in human HeLa cells impaired the formation of 53BP1 foci (Yang, et al., 2008), suggesting that dimethyl H4K20 is also required for a proper DNA damage response in human cells. The proposed mechanism involves exposure of normally buried H4K20me2 epitopes upon DNA double strand breaks (Yang and Mizzen, 2009; Botuyan, et al., 2006).

Histone H4 lysine 20 trimethylation (H4K20me3) has been implicated in the formation of constitutive heterochromatin, particularly at (peri)centromeric and (sub)telomeric regions (Schotta, et al., 2004; Benetti, et al., 2007; Kourmouli, et al., 2004; Sims, et al., 2006). This requires trimethylation of H3K9 by Suv39h1/2 enzymes and the subsequent binding of HP1 proteins, which function to recruit Suv420h1/2 (Schotta, et al., 2004). Accordingly, trimethylation of H4K20, but not dimethylation, is reduced in Drosophila larvae lacking HP1 (Yang, et al., 2008).

H4K20me3 has been shown to be down-regulated in human cancer (Fraga, et al., 2005; Van Den Broeck, et al., 2008). This might be achieved via downregulation of the Suv420h1/2 enzymes (Van Den Broeck, et al., 2008; Pogribny, et al., 2006; Tryndyak, et al., 2006), but also by alteration of HP1 binding dynamics (Siddiqui, et al., 2007). Loss of H4K20me3 might lead to activation of transcription of previously repressed genes (through alteration of chromatin structure), unstable kinetochore attachment sites, as well as uncapped telomeres (Benetti, et al., 2007a; 2007b). In a normal cell, these effects might lead to cell cycle arrest and/or programmed cell death; however, in a malignant cell, this might only serve to increase genomic instability, contributing to further tumorigenesis. In addition, downregulation of the Suv420h enzymes also impairs DNA damage signaling through loss of H4K20me2, further contributing to genomic instability (Schotta, et al., 2008; Rouse and Jackson, 2002; Zhou and Elledge, 2000).

We found that apoptin interacts with Suv420h1 in human tumor cells, and both proteins seem to cooperate to induce apoptosis in these cells (R. Zimmerman, unpublished results). Preliminary results suggest that Suv420h1 itself was also able to induce tumor-selective apoptosis, as its over-expression induced apoptosis in tumor cells but not in normal cells. Furthermore, we found that Suv420h1 interacted with HP1 beta in both normal and cancer cells (R. Zimmerman and H. Lanz, unpublished results). This concurs with reports that HP1 beta is involved in DNA damage signaling originating from DNA breaks in H3K9me3-containing chromatin (Ayoub, et al., 2008).

*BCA3/AKIP1 (**Chapter 6**)*

Another protein identified as an interacting partner of apoptin in our yeast two-hybrid assay is BCA3 (**chapter 6**). Breast cancer associated gene 3 (BCA3) was identified as a gene that was overexpressed in breast cancer compared to surrounding normal stroma (Kitching, et al., 2003; Leon and Canaves, 2003). It has been shown to interact with a number of proteins, including the catalytic subunit of protein kinase A (hence the alternative name A-kinase interacting protein 1, or AKIP1) (Sastri, et al., 2005), the transcription factor KyoT2 (Qin, et al., 2004), and Rac1 (Yu, et al., 2007).

Under normal cellular conditions, PKA resides in the cytoplasm as an inactive holoenzyme consisting of two regulatory subunits complexed with two catalytic subunits. A-kinase associated proteins, or AKAPs, interact with the regulatory subunits and control PKA activity by anchoring the holoenzyme complexes at specific subcellular localizations. Upon activation by cAMP, the regulatory subunits dissociate, freeing the catalytic subunits. AKIP1 binds to the latter, reportedly facilitating its translocation into the nucleus (Sastri, et al., 2005).

Over-expression of BCA3 appeared to induce apoptosis in human tumor cells, and co-expression with apoptin led to an increase in tumor cell death (**Chapter 6**). The negative effect of BCA3 on cellular proliferation had been

observed previously in osteoclasts (Yu, et al., 2007). In these cells, it was shown to interact with Rac1, a small GTPase that belongs to the Ras superfamily and participates in a wide range of biological processes, including rearrangement of the actin cytoskeleton, cell motility, cell transformation, gene transcription, and cell cycle progression (Michiels and Collard, 1999; Hall, 2005). The interaction of BCA3 with Rac1 attenuated colony stimulating factor-1 (CSF-1) induced cell spreading.

Another interaction partner of BCA3 was identified as TAp73 (Leung and Ngan, 2010). TAp73 is highly similar to p53, and exhibits growth-inhibitory, tumor-suppressive, and proapoptotic functions (Kaghad, et al., 1997; Jost, et al., 1997). TAp73 binds and stabilizes BCA3 in the cervical cancer cell line HeLa (Leung and Ngan, 2010). When coexpressed with TAp73, BCA3 interacts and colocalizes with TAp73 at the mitochondria. Furthermore, BCA3 augments the transactivation activity of TAp73 on Bax promoter, enhancing Bax protein expression. In addition, the expression of BCA3 also increases the sensitivity of TAp73-transfected cells to γ-irradiation–induced apoptosis, inducing activation of caspase-7 and caspase-9. Interestingly, Klanrit et al. (2008) reported that over-expression of TAp73 was able to sensitize p53-negative tumor cells to apoptin-induced cell death.

APC

In transformed cells but not in normal cells, apoptin associates with APC1, the largest subunit of the anaphase-promoting complex/cyclosome (APC/C) (Teodoro, et al., 2004) and an essential component of the mitotic checkpoint apparatus (see **chapter 2**). Apoptin expression, or depletion of APC1 by RNA interference, inhibits APC/C function in p53 null cells, resulting in G2/M arrest and apoptosis. Furthermore, apoptin expression in transformed cells induces the formation of nuclear bodies and recruits APC/C to these subnuclear structures (Heilman, et al., 2006); the interaction with PML is, however, not required for induction of apoptosis by apoptin (Janssen, et al., 2007).

Teodoro, et al. (2004) also reported the interaction of apoptin with α-tubulin, β-tubulin and β-actin, suggesting an association with filamentous networks. As subunits of the APC/C, including APC1, have been shown to localize to the active centromere of dicentric chromosomes (Saffery, et al., 2000), they proposed that the observed association of apoptin with α- and β-tubulin might result from interaction between apoptin and the spindle complex. The interaction of apoptin with FAM96B (which controls chromosome segregation, **chapter 4**) and BCA3 (which localizes to the centrosome, **chapter 6**), as well as the identification of tubulin in chromatin-bound apoptin complexes (**chapter 5**), indeed appears to be in agreement with this suggestion.

Apoptin phosphorylation

Alas, the list of apoptin interacting proteins lacks one crucial protein: a kinase, which is able to phosphorylate apoptin at T108, and which is only active in transformed and tumor cells. For, though they were found to interact with apoptin, neither PI3K nor PKA were able to phosphorylate it.

Maddika, et al. (2009) described that Cdk2 complexed with cyclin A, but not E, could phosphorylate apoptin at T108, yet could not prove that this phosphorylation was specific to the tumorigenic environment. PKCβ has also been implicated in apoptin phosphorylation (Jiang, et al., 2010), Recently, however, our laboratory showed that that immunoprecipitation of both CDK2 and PKCβ significantly reduced the levels of these proteins in tumor cell lysates, without affecting the level of apoptin phosphorylation by these tumor cell lysates in an *in vitro* kinase assay (Lanz, et al., 2012). Therefore, at the moment no conclusive statement can be drawn about the kinase modifying and activating apoptin.

Zhang, et al. (2004) did find that the apoptin kinase activity could be specifically turned on by transient transformation of normal human fibroblasts expressing apoptin with the SV40 large T antigen. These results were further investigated in **chapter 6**. Here, we demonstrated that upon

expression of the SV40 large T antigen (LT), the small T antigen (ST) was also expressed. In fact, co-expression of the ST was essential to induce apoptin phosphorylation upon cellular transformation by SV40 LT. Analysis of ST functional domains, through stepwise mutational inactivation, proved that the interaction of ST with B56 gamma-containing PP2A was required for apoptin activation. Simultaneously, analysis of the effect of PKA activity on apoptin phosphorylation pointed to its involvement in the regulation of PP2A B56 delta activity. Subsequently, we found that down-regulation of PP2A B56 gamma and B56 delta expression through RNAi in normal cells indeed resulted in apoptin phosphorylation. Hence, the data presented in this thesis argue for an apoptin normal-cell-specific phosphatase next to a tumor-specific kinase, even not excluding the possibility that apoptin might be phosphorylated by more than one kinase, and that its differential activation between normal and tumor cells rather depends on the activity of the tumor suppressor phosphatase PP2A.

Outlook

Synthesis – how might the pieces of the apoptin puzzle fit together?

The experimental results with the newly discovered apoptin-interacting proteins presented in this thesis, combined with knowledge from previously published analyses, lead to the proposal of the following model for apoptin behavior in the human cell (figure 7.2).

In the normal human cell (figure 7.2A), upon expression of the apoptin protein, apoptin is at first free to move between the nucleus and cytoplasm, owing to its NLS and NES. Apoptin might be phosphorylated, but it is also dephosphorylated by PP2A, reaching an equilibrium which finds it mostly unphosphorylated. Apoptin is mainly located in the cytoplasm, where it interacts with Hippi and accumulates in cytoplasmic granules. The exact nature of these cytoplasmic granules is not known; immune fluorescence analysis has revealed partial co-localization with markers for the lysosome, Golgi, ER and endosomal vesicles as well (R. Zimmerman, unpublished

results). Whichever the mechanism, apoptin does in fact disappear from the normal cell, without inducing cell death.

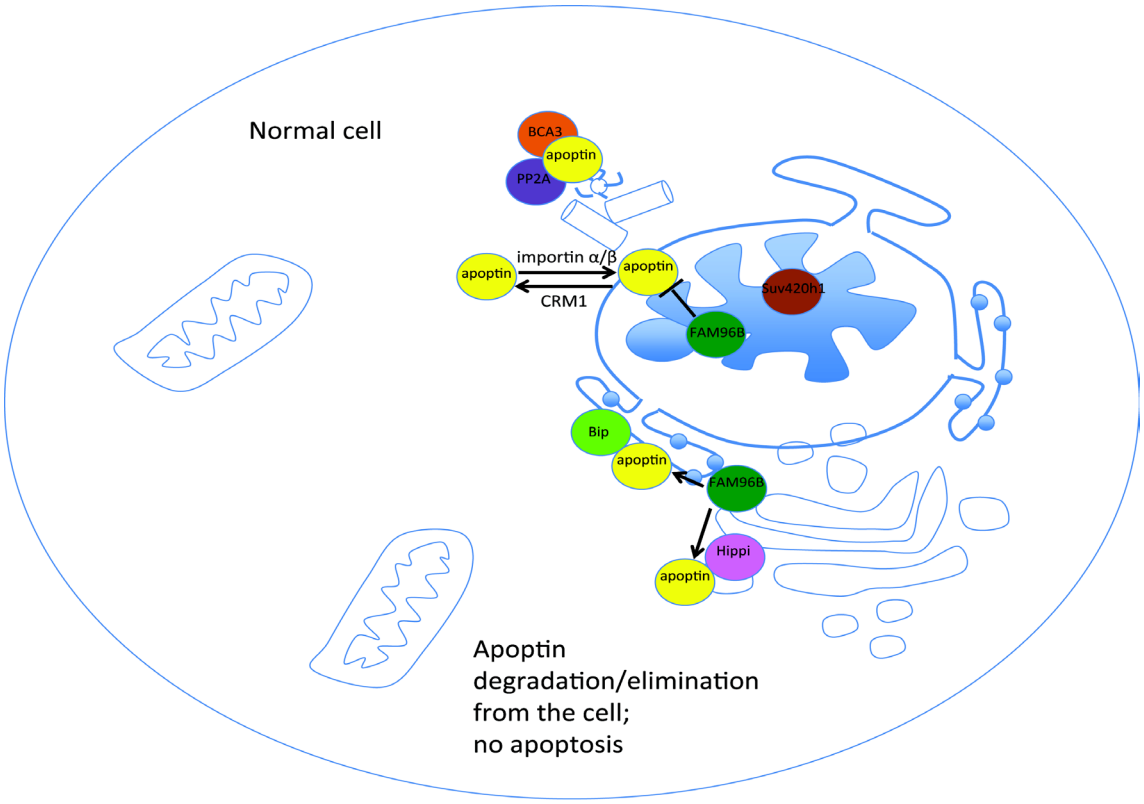

Figure 7.2 Model of apoptin tumor-specific cell death pathways. **A.** In the normal cell, apoptin phosphorylation is opposed by the action of PP2A B56 gamma- and delta-containing complexes. The presence of FAM96B safeguards the cell cycle, while H4K20 di- and trimethylation function to recruit DNA repair machinery and telomere-capping proteins, respectively, preventing genomic instability. As a result of these actions, apoptin is redirected to the cytoplasm, where it interacts with e.g. Bip in the ER and Hippi in Golgi vesicles, and is eventually eliminated from the cell without inducing apoptosis. See text for further details.

In the transformed cell (figure 7.2B), many cellular processes have gone awry: the activity of (potential) tumor suppressor proteins PP2A, FAM96B and Suv420h1 is derailed, while the expression of (potentially) oncogenic proteins, Rsf-1 and BCA3 has increased. Loss of FAM96B results in a failure to arrest the cell cycle upon DNA damage, whereas loss of H4K20 trimethylation results in telomere uncapping and lack of proper pericentric heterochromatin formation, resulting in inappropriate kinetochore attachment sites. Over-expression of Rsf-1 and BCA3 might also contribute to the formation of aberrant mitotic spindles; altogether, the resulting

genomic instability would constantly signal to the DNA damage response machinery, whereas the ability to arrest the cell cycle and/or induce apoptosis has been lost. Loss of PP2A activity means apoptin phosphorylation is no longer opposed, leading to the accumulation of apoptin in the tumor cell nucleus. Within the nucleus, apoptin localizes to the nucleoli, where it cooperates with Rybp/DEDAF and e.g. nucleophosmin in sensing the many DNA damage signals. Furthermore, apoptin interacts with Rsf-1 at chromosome centromeres, where it inhibits APC1 activity. As a result, the APC cyclosome cannot catalyze chromosome segregation; in addition, interference of apoptin and BCA3 with the mitotic spindle contributes to the resulting mitotic failure and the inevitable cell death following it (figure 7.2 C, D).

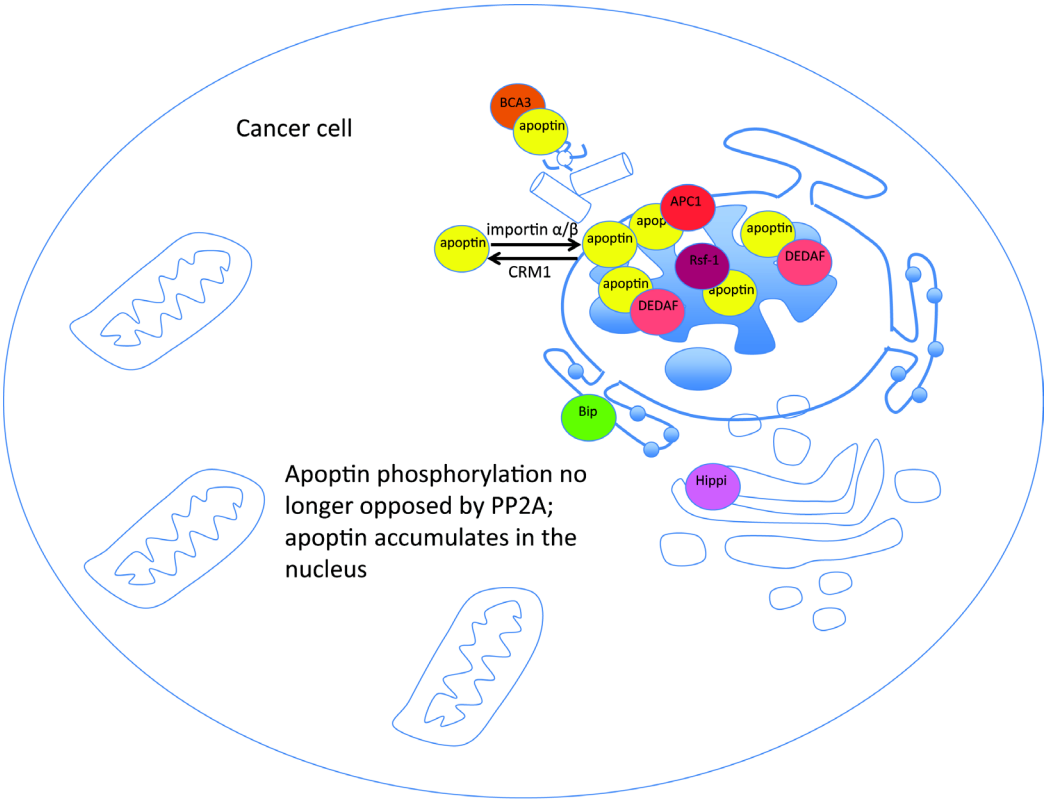

Figure 7.2 Model of apoptin tumor-specific cell death pathways. **B.** In the cancer cell, apoptin phosphorylation is no longer opposed, and phosphorylated apoptin accumulates in the nucleus, colocalizing with DEDAF and other proteins in the nucleolus, while also colocalizing with BCA3 at the centrosome. See text for further details.

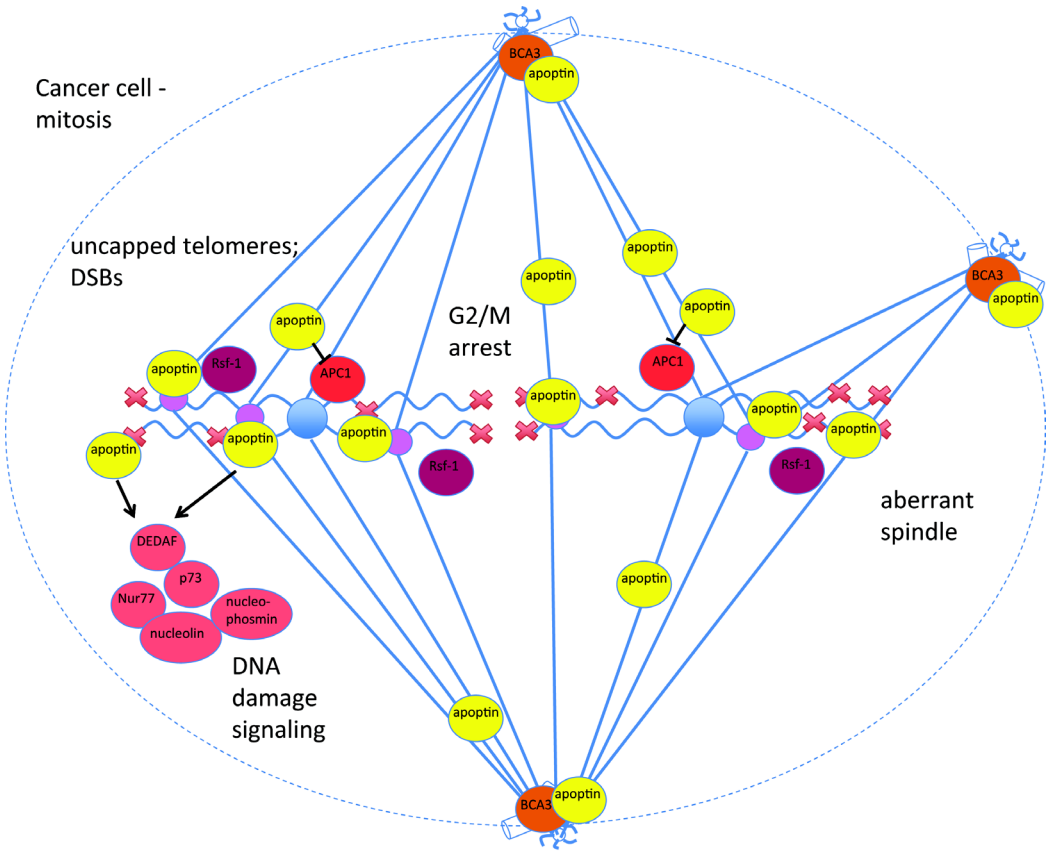

Figure 7.2 Model of apoptin tumor-specific cell death pathways. **C.** Over-expression of Rsf-1 and BCA3 contribute to the formation of faulty mitotic spindles. Lack of H4K20 methylation means DSBs are no longer repaired, and telomeres become uncapped. Apoptin recognizes the activation of the DNA damage response, and inhibits APC1 activity, halting the anaphase-promoting complex and inducing G2/M arrest. See text for further details.

How apoptin might distinguish between normal and tumor cells

From the proposed model, it results that apoptin's differential recognition of normal and transformed cells likely depends on three key properties of malignant cells: (1) sustained cellular proliferation and (2) the lack of execution of programmed cell death in the face of (3) severe DNA damage. Though apoptin has been shown to possess two different death domains, one acting from within the cytoplasm (Danen-van Oorschot, et al., 2003), it seems that the crucial step in blocking apoptin-induced apoptosis in normal cells is inhibiting its nuclear accumulation. Still, even if apoptin is forced to the nucleus of normal cells, it has been confirmed that this is not enough to induce apoptosis (Danen-van Oorschot, et al., 2003) – probably because of the lack of signals that cellular proliferation has gone awry, including silencing of tumor suppressor genes (e.g. FAM96B, PP2A), over-expression of

oncogenes (e.g. Rsf-1, and possibly BCA3), and the lack of DNA damage. It is the combination of these events that appears to tip the equilibrium to the side of apoptin phosphorylation, and hence, activation. Indeed, recent experiments (Kucharski, et al., 2011) demonstrated that induction of DNA damage was sufficient to activate apoptin, resulting in its nuclear translocation and induction of apoptosis in primary cells.

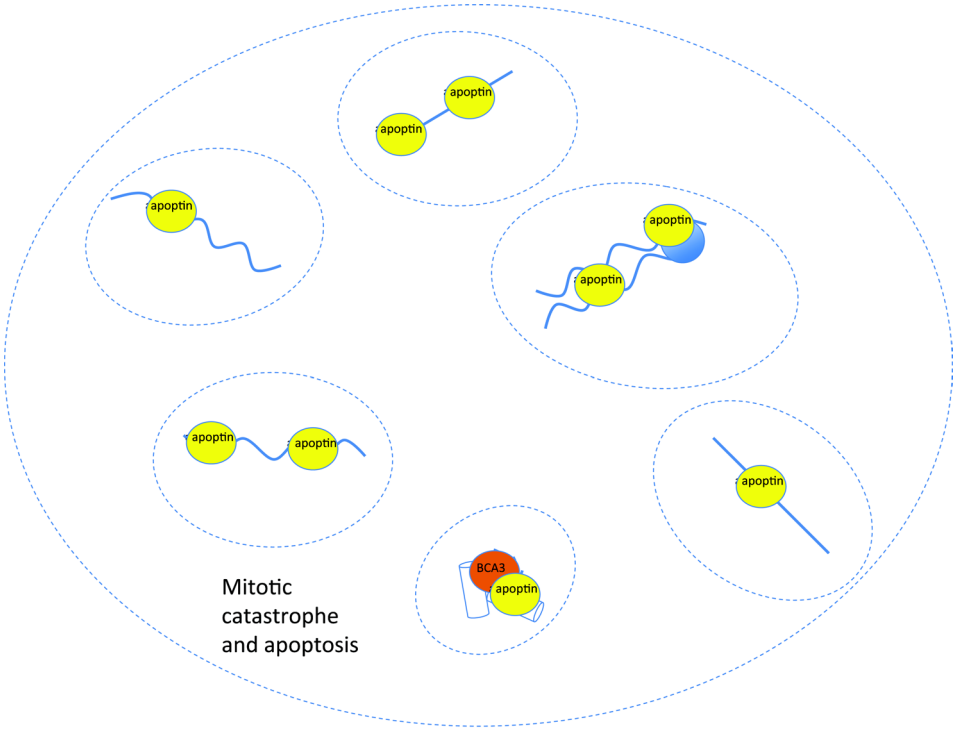

Figure 7.2 Model of apoptin tumor-specific cell death pathways. **D.** These effects, in addition to apoptin association with the mitotic spindle, lead to mitotic catastrophe and apoptotic cell death. See text for further details.

Mechanism of apoptin-induced apoptosis in tumor cells

In the model proposed above, activated apoptin induces cell death through several modes of action: i) it localizes to the nucleoli, where it ia) senses DNA damage, activating DEDAF, among other proteins, to induce apoptosis, and ib) interacts with ribosomal chromatin and constituents, effectively shutting off cellular biosynthesis; ii) it induces mitotic failure through a doubly lethal combination of iia) interaction cq interference with several components of the mitotic spindle; and iib) inhibition of APC-catalyzed segregation of chromosomes. Not only is the cell therefore physically unable to divide,

leading to mitotic catastrophe, but also, even if the cell would manage to survive this event, it would die as a result of the lack of cellular biosynthesis.

The results described in this thesis correlate with the previous findings in literature, stating that apoptin-induced tumor-selective apoptosis does not require *de novo* gene transcription/translation (Danen-van Oorschot, et al., 2003). The same is true for the independence of p53 activity (Zhuang, et al., 1995b), and the paradoxical relation with Bcl-2. As discussed in **chapter 2**, during mitosis, DNA damage activates the ATM/ATR signaling pathways; DNA single strand breaks (SSBs) activate Chk1, which inhibits the action of Cdc25c, leading to G2/M cell cycle arrest – as previously observed for apoptin, and independently of p53. DNA double strand breaks (DSBs) activate Chk2, which activates both p53 and p73; intriguingly, p73 has indeed been shown to be involved in apoptin-induced apoptosis. Furthermore, DNA damage-induced signaling, through an as yet unclear mechanism, leads to the activation of caspase-2, which directly stimulates cytochrome c release, G2/M cell cycle arrest and mitotic catastrophe, independently of either p53 or Bcl-2. In fact, it has been reported that over-expression of Bcl-2 resulted in an enhanced frequency of mitotic catastrophe (see **chapter 2**). This is consistent with reports that over-expression of Bcl-2 does not necessarily impede apoptin-induced apoptosis, and may even have a stimulatory effect (Danen-Van Oorschot, et al., 1999; Danen-Van Oorschot, et al., 1999b).

Implications for CAV pathogenesis

One cannot help but wonder how all this applies to the natural function of apoptin in CAV pathogenesis. In young chicks, CAV has been shown to preferentially target the cells of the bone marrow and thymus (Adair, 2000); besides rapid proliferation, these cells are also constantly undergoing rearrangements of their DNA, which could be perceived by apoptin as damaged DNA. In addition, many viral proteins have been shown to localize to the nucleolus (Hiscox, 2007), where they hijack the cell's protein factory and redirect it towards their own multiplication. The subsequent induction

of apoptosis then leads to release of the viral progeny, and, concurrently, to further spreading of the infection, as nearby cells engulf the apoptotic bodies containing newly formed viral particles.

Chickens develop a resistance to CAV-induced disease by two weeks of age; furthermore, experiments in normal human lymphocytes have shown that apoptin is not toxic to these cells. In addition, systemic expression of apoptin in mice does not induce apoptosis in erythroid or lymphogenic cells or their precursors, indicating that apoptin is indeed safe for anti-tumor therapy in humans (Pietersen, et al., 2005; Peng, et al., 2007).

Implications for anticancer therapies

A comparison between apoptin and the other PKTC presented in **chapter 3** reveals a number of common strategies, which could be exploited in the design of novel anticancer therapies.

Apoptin (Liu, et al., 2006a, 2006b), TRAIL (Voelkel-Johnson, et al., 2005) and MDA-7/IL-24 (Sauane, et al., 2010) have all been shown to up-regulate ceramide production, indicating that this might be a convenient strategy in killing cancer cells. Autophagy seems to be a common theme, with HAMLET (Aits, et al., 2009), NS1 (Bruno, et al., 2009), and Brevinin-2R (Ghavami, et al., 2008) all employing autophagic cell death to specifically kill tumor cells. As one of the pathways mitigating the metabolic switch (the seventh cancer hallmark), this indeed seems warranted. Perhaps related to this, the adenovirus E4orf4 protein has been demonstrated to kill tumor cells by perturbing the traffic of endosomal vesicles (Landry, et al., 2009). E4orf4-induced cell death relies on Src tyrosine kinases and RhoGTPase-dependent perturbation of actin dynamics (Robert, et al., 2006); similarly, apoptin has also been shown to target the cytoskeleton and mitotic spindle (**chapters 4-6**), and both apoptin (Teodoro, et al., 2004) and E4orf4 (Kornitzer, et al., 2001) have been shown to induce G2/M cell cycle arrest by targeting the APC/C anaphase promoting complex. With respect to the various microtubule-destabilizing agents that are now being used, the APC/C

cyclosome might well represent a novel target for the development of cancer-combating agents (Heilman, et al., 2005).

The interaction between apoptin and Rsf-1 and Suv420h1 (A. Danen-van Oorschot and R. Zimmerman, unpublished results) suggests employing molecules that target epigenetic chromatin modifiers. Besides the inhibitors of histone deacetylases (HDACs), which are already being used, alteration of the activities of histone methylases and chromatin remodeling complexes could either limit genomic instability and thus tumor progression, or, alternatively, promote genomic instability beyond a point compatible with life. The over-expression of Rsf-1 in tumor cells has also made it an attractive target for immunotherapies. In fact, transduction of dendritic cells with plasmid DNA encoding the Rsf-1 gene demonstrably generated specific cytotoxic T lymphocytes against ovarian cancer *in vitro* (Sun, et al., 2010).

In **chapter 5**, we inferred the localization of apoptin in the nucleoli of cancer cells, which in fact are the ribosome factories facilitating the rapid cell growth characteristic of tumor cells. Thus, complementary to the proteasome inhibitors already employed in current anticancer therapies, shutting off excessive ribosomal production might also prove an effective strategy in the fight against cancer.

Conclusions

As discussed in **chapter 2**, the tumor-killing potential of the apoptin protein has already been established *in vivo*, as demonstrated by experiments in our laboratory with apoptin-producing adenovirus (Pietersen, et al., 1999), Asor-apoptin (Peng, et al., 2007) and PTD4-apoptin (Sun, et al., 2009; Jin, et al., 2011), all in which apoptin reduced tumor cell mass and prolonged survival of the affected organism, without harming normal cells.

In this thesis, the molecular aspects of apoptin-induced tumor-selective apoptosis have been investigated. The basis for apoptin's tumor-selectivity has been further deciphered, as well as the mechanisms leading to apoptin-

induced apoptosis in malignant cells. These involve the recognition by apoptin of several of the cancer hallmarks, including:

- genomic instability (apoptin senses DNA damage through e.g. Rybp/DEDAF, and telomere uncapping by loss of H4K20 trimethylation).
- insensitivity to inhibitory cell cycle checkpoints (lack of FAM96B, aberrant chromatin and spindle structures through interaction with overexpressed Rsf-1 and BCA3);
- evasion of programmed cell death (apoptin-induced apoptosis is stimulated by over-expression of Bcl-2);
- activation of the metabolic switch (apoptin interacts with PI3K and Akt)

Thus, recognition of malignant transformation by apoptin occurs already in the early stages of transformation, before the acquisition of angiogenic, metastatic and immune evasive potential, and possibly even before activation of telomere maintenance and cellular immortalization. As described above, apoptin also utilizes these cancer hallmarks to attack the tumor cell at various points and bring about cell death.

It seems that apoptin, but also the other PKTC presented in **chapter 3**, employ several different strategies to ensure death of the cancer cell. Considering this, as well as the fact that cancer cells, owing to their propensity to rapidly accumulate genetic changes, are essentially moving targets, it might be naïve to assume that we can win the fight against cancer by employing a single strategy. Thus, rather than trying to devise a magic bullet, we might instead compose a magical cluster bomb, containing subparticles aimed at each of the above-described tumor-specific targets, attacking cancer from every conceivable angle, and attaining cell death, without the harmful side-effects of radiation and conventional chemotherapy.

Table 7-1 **Summary of apoptin-associating proteins.**

Protein	**Cellular function**	**Subcellular localization**	**Expression in cancer**	**Involvement in apoptin activity**	**References**
Rybp/DEDAF	Transcription regulation; DNA damage response; Apoptosis	Predominantly nuclear	Decreased	Co-expression stimulates apoptosis	*See references in text*
Rsf-1	Chromatin remodeling	Nuclear	Increased	Co-expression stimulates apoptosis	*See references in text*
FAM96B	Sister chromatid cohesion; Cell cycle regulation	Nuclear + cytoplasmic	Decreased	Co-expression inhibits apoptosis	*See references in text*
Suv420h1	Histone H4 lysine 20 di- and trimethylation	Nuclear	Possibly decreased	Co-expression stimulates apoptosis	*See references in text*
BCA3/AKIP1	Regulation of transcription and possibly cytoskeleton dynamics	Perinuclear	Increased	Co-expression stimulates apoptosis	*See references in text*
Bip/GRP78/HSPA5	Protein chaperone; mediator of unfolded protein response and ER stress	Endoplasmic reticulum	Increased	Unknown	Sato, et al., 2010; Teodoro, et al., 2004; Danen-van Oorschot and Zimmerman, unpublished results

Table 7-1 **continued.**

Protein	**Cellular function**	**Subcellular localization**	**Expression in cancer**	**Involvement in apoptin activity**	**References**
Nmi	Transcription regulation	Primarily cytosolic	Increased	Uknown	Sun, et al., 2002; Zhou, et al., 2000 Danen-van Oorschot and Zimmerman, unpublished results
Hippi	Intracellular transport; Apoptosis	Golgi	Unknown	Possibly involved in apoptin elimination from the normal cell	*See references in text*
Ppil3	Protein-folding chaperone catalyzing cis-trans peptidylprolyl somerisation	Nuclear + cytoplasmic	Possibly decreased	Co-expression enhances apoptin cytoplasmic localization in cancer, but has no effect on apoptosis activity	Huo, Yi, & Yang, 2008
APC1	E3 ubiquitin ligase; subunit of anaphase-promoting complex	Nuclear + cytoplasmic	Unknown	Inhibition by apoptin results in G2/M arrest and apoptosis	*See references in text*
PML	Transcription regulation; DNA damage response	Nucleolus; PML bodies	Increased; decreased in advanced stages	Interaction not essential for apoptosis induction	*See references in text*

Table 7-1 **continued.**

Protein	Cellular function	Subcellular localization	Expression in cancer	Involvement in apoptin activity	References
PKAc	Catalytic subunit of cAMP-dependent protein kinase, involved in many intracellular signalling patways, including those stimulating proliferation and survival	Various compartments, dependent on association with AKAPs	Various isoforms up-regulated	Stimulation reduces apoptin phosphorylation	*See references in text*
p85	Regulatory subunit of PI3K, a kinase strongly implicated in tumorigenesis, with functions in proliferation, survival, angiogenesis, autophagy.	Cytoplasmic	Activating gain-of-function mutations	Activation stimulates apoptin-induced apoptosis	*See references in text*
Akt	Downstream effector of PI3K signalling	Nuclear + cytoplasmic	Increased	Co-expression of nuclear Akt stimulates apoptin-induced apoptosis	*See references in text*
Cdk2/cyclin A	Promotes G1/S transition and replication of DNA and centrosomes in S-phase	Nuclear + cytoplasmic	Unknown	Phosphorylates apoptin directly *in vitro*	*See references in text*

Table 7-1 **continued.**

Protein	**Cellular function**	**Subcellular localization**	**Expression in cancer**	**Involvement in apoptin activity**	**References**
PKCβ	Diverse intracellular signaling pathways, including transformation, proliferation, (inhibition of) apoptosis	Nuclear + Cytoplasmic	Unclear, though increased expression is correlated with poorer prognosis	Phosphorylates apoptin directly *in vitro*	*See references in text*
PP2A B56γ	Protein phosphatase with important tumor suppressor functions, including regulation of DNA damage response and several stages of cell cycle progression	Nuclear, centromeric	Decreased/inactivated in cancer	Dephosphorylates apoptin directly *in vitro*	*See references in text*

***AKAPs, A-kinase anchoring proteins.**

References

Abrams S, Lakum T, Lin K, Jones G, Treweeke A, Farahani M et al. (2007) B-cell receptor signaling in chronic lymphocytic leukemia cells is regulated by overexpressed active protein kinase CbetaII. *Blood* 109, 1193-1201.

Adair B (2000) Immunopathogenesis of chicken anemia virus infection. *Dev Comp Immunol* 24, 247-255.

Aits S, Gustafsson L, Hallgren O, Brest P, Gustafsson M, Trulsson M et al. (2009) HAMLET (human alpha-lactalbumin made lethal to tumor cells) triggers autophagic tumor cell death. *Int J Cancer* 124, 1008-1019.

Arroyo J and Hahn W (2005) Involvement of PP2A in viral and cellular transformation. *Oncogene* 24, 7746-7755.

Ayoub N, Jeyasekharan A, Bernal J and Venkitaraman A (2008) HP1-beta mobilization promotes chromatin changes that initiate the DNA damage response. *Nature* 453, 682-686.

Backendorf C, Visser A, De Boer A, Zimmerman R, Visser M, Voskamp P et al. (2008) Apoptin: Therapeutic potential of an early sensor of carcinogenic transformation. *Annu Rev Pharmacol Toxicol* 48, 143-169.

Bejarano F, Gonzalez I, Vidal M and Busturia A (2005) The Drosophila RYBP gene functions as a Polycomb-dependent transcriptional repressor. *Mech Dev* 122, 1118-1129.

Ben-Aroya S, Coombes C, Kwok T, O'Donnell K, Boeke J and Hieter P (2008). Toward a comprehensive temperature-sensitive mutant repository of the essential genes of Saccharomyces cerevisiae. *Molecular Cell* 30, 248.

Benetti R, García-Cao M and Blasco M (2007) Telomere length regulates the epigenetic status of mammalian telomeres and subtelomeres. *Nat Genet* 39, 243-250.

Benetti R, Gonzalo S, Jaco I, Schotta G, Klatt P, Jenuwein T et al. (2007) Suv4-20h deficiency results in telomere elongation and derepression of telomere recombination. *J Cell Biol* 178, 925-936.

Borg A, Tandon A, Sigurdsson H, Clark, G, Fernö M, Fuqua S et al. (1990) HER-2/neu amplification predicts poor survival in node-positive breast cancer. *Cancer Res* 50, 4332-4337.

Botuyan M, Lee J, Ward I, Kim J, Thompson J, Chen J et al. (2006) Structural basis for the methylation state-specific recognition of histone H4-K20 by 53BP1 and Crb2 in DNA repair. *Cell* 127, 1361-1373.

Bruno P, Brinkmann C, Boulanger M, Flinterman M, Klanrit P, Landry M et al. (2009) Family at last: highlights of the first international meeting on proteins killing tumour cells. *Cell Death and Differentiation* 16, 184-186.

Chen D, Zhang J, Li M, Rayburn E, Wang H and Zhang R (2009) RYBP stabilizes p53 by modulating MDM2. *EMBO Rep* 10, 166-172.

Cheng C, Huang S, Chang Y, Chung W and Yuo C (2003) The viral death protein Apoptin interacts with Hippi, the protein interactor of Huntingtin-interacting protein 1. *Biochem Biophys Res Commun* 305, 359-364.

Choi J, Sheu J, Guan B, Jinawath N, Markowski P, Wang T et al. (2009) Functional analysis of 11q13.5 amplicon identifies Rsf-1 (HBXAP) as a gene involved in paclitaxel resistance in ovarian cancer. *Cancer Res* 69, 1407-1415.

Coffer P, Jin J and Woodgett J (1998) Protein kinase B (c-Akt): a multifunctional mediator of phosphatidylinositol 3-kinase activation. *Biochem J* 335, 1-13.

Cosma M, Tanaka T and Nasmyth K (1999) Ordered recruitment of transcription and chromatin remodeling factors to a cell cycle- and developmentally regulated promoter. *Cell* 97, 299-311.

Cui X, De Vivo I, Slany R, Miyamoto A, Firestein R and Cleary M (1998) Association of SET domain and myotubularin-related proteins modulates growth control. *Nat Genet* 18, 331-337.

Danen-van Oorschot A, Fischer D, Grimbergen J, Klein B, Zhuang S-M, Falkenburg J et al. (1997) Apoptin induces apoptosis in human transformed and malignant cells but not in normal cells. *Proc Natl Acad Sci U S A* 94, 5843-5847.

Danen-Van Oorschot A, van der Eb A and Noteborn M (1999) BCL-2 stimulates Apoptin-induced apoptosis. *Adv Exp Med Biol* 457, 245-249.

Danen-van Oorschot A, Voskamp P, Seelen M, van Miltenburg M, Bolk M, Tait S et al. (2004) Human death effector domain-associated factor interacts with the viral apoptosis agonist Apoptin and exerts tumor-preferential cell killing. *Cell Death Differ* 11, 564-573.

Danen-van Oorschot A, Zhang Y-H, Leliveld S, Rohn J, Seelen M, Bolk M et al. (2003) Importance of nuclear localization of apoptin for tumor-specific induction of apotosis. *J Biol Chem* 278, 27729-27736.

Danen-Van Oorschot A, Zhang Y-H, Erkeland S, Fischer D, van der Eb A and Noteborn M (1999) The effect of Bcl-2 on Apoptin in 'normal' vs transformed human cells. *Leukemia* 13, S75-S77.

De Vivo I, Cui X, Domen J and Cleary M (1998) Growth stimulation of primary B cell precursors by the anti-phosphatase Sbf1. *Proc Natl Acad Sci U S A* 95, 9471-9476.

Eichhorn P, Creyghton M and Bernards R (2009) Protein phosphatase 2A regulatory subunits and cancer. *Biochim Biophys Acta* 1795, 1-15.

Ferrier V (2002) Hip, hip, hippi! *Nat Cell Biol* 4, E30.

Finn R, Mistry J, Tate J, Coggill P, Heger A, Pollington J et al. (2010) The Pfam protein families database. *Nucleic Acids Res* 38, D211-222.

Flanagan J and Peterson C (1999) A role for the yeast SWI/SNF complex in DNA replication. *Nucleic Acids Res* 27, 2022-2028.

Fraga M, Ballestar E, Villar-Garea A, Boix-Chornet M, Espada J, Schotta G et al. (2005) Loss of acetylation at Lys16 and trimethylation at Lys20 of histone H4 is a common hallmark of human cancer. *Nat Genet* 37, 391-400.

Fruman D, Meyers R and Cantley L (1998) Phosphoinositide kinases. *Annu Rev Biochem* 67, 481–507.

Garcia E, Marcos-Gutierrez C, del Mar Lorente M, Moreno J and Vidal M (1999) RYBP, a new repressor protein that interacts with components of the mammalian Polycomb complex, and with the transcription factor YY1. *EMBO J* 18, 3404–3418.

Gervais F, Singaraja R, Xanthoudakis S, Gutekunst C, Leavitt B, Metzler M et al. (2002) Recruitment and activation of Caspase-8 by the Huntingtin-interacting protein Hip-1 and a novel partner Hippi. *Nat Cell Biol 4*, 95-105.

Ghavami S, Asoodeh A, Klonisch T, Halayko A, Kadkhoda K, Kroczak T et al. (2008) Brevinin-2R(1) semi-selectively kills cancer cells by a distinct mechanism, which involves the lysosomal-mitochondrial death pathway. *J Cell Mol Med* 12, 1005-1022.

Greaves K, Rangasamy D, Devoy M, Marshall Graves A and Tremethick J (2006) The X and Y chromosomes assemble into H2A.Z, containing facultive heterochromatin, following meiosis. *Mol Cell Biol* 26, 5394–5405.

Greaves K, Rangasamy D, Ridgway P and Tremethick J (2007) H2A.Z contributes to the unique 3D structure of the centromere. *Proc Natl Acad Sci U S A* 104, 525–530.

Greeson N, Sengupta R, Arida A, Jenuwein T and Sanders S (2008) Di-methyl H4 lysine 20 targets the checkpoint protein Crb2 to sites of DNA damage. *J Biol Chem 283*, 33168-33174.

Hall A (2005) Rho GTPases and the control of cell behaviour. *Biochem Soc Trans 33*, 891-895.

Hanai K, Furuhashi H, Yamamoto T, Akasaka K and Hirose S (2008) RSF governs silent chromatin formation via histone H2Av replacement. *PLoS Genet* 4, e1000011.

Heilman D, Green M and Teodoro J (2005) The anaphase promoting complex: a critical target for viral proteins and anti-cancer drugs. *Cell Cycle* 4, 560-563.

Hiscox J (2007) RNA viruses: hijacking the dynamic nucleolus. *Nat Rev Microbiol* 5, 119-127.

Huang W, Batra S, Atkins B, Mishra V and Mehta K (2005) Increases in intracellular calcium dephosphorylate histone H3 at serine 10 in human hepatoma cells: potential role of protein phosphatase 2A-protein kinase CbetaII complex. *J Cell Physiol* 205, 37-46.

Huo D, Yi L and Yang J (2008) Interaction with Ppil3 leads to the cytoplasmic localization of Apoptin in tumor cells. *Biochem Biophys Res Commun* 372, 14-18.

Ito S, Tan L, Andoh D, Narita T, Seki M, Hirano Y et al. (2010) MMXD, a TFIIH-independent XPD-MMS19 protein complex involved in chromosome segregation. *Mol Cell* 39, 632-640.

Janssen K, Hofmann T, Jans D, Hay R, Schulze-Osthoff K and Fischer U (2007) Apoptin is modified by SUMO conjugation and targeted to promyelocytic leukemia protein nuclear bodies. *Oncogene* 26, 1557-1566.

Jeurissen S, Wagenaar F, Pol J, van der Eb A and Noteborn M (1992) Chicken anemia virus causes apoptosis of thymocytes after *in vivo* infection and of cell lines after *in vitro* infection. *J Virol 66*, 7383–7388.

Jiang J, Cole D, Westwood N, Macpherson L, Farzaneh F, Mufti G et al. (2010) Crucial roles for protein kinase C isoforms in tumor-specific killing by Apoptin. *Cancer Res* 70, 7242-7252.

Jin J, Gong J, Yin T, Lu Y, Xia J, Xie Y et al. (2011). PTD4-apoptin protein and dacarbazine show a synergistic antitumor effect on B16-F1 melanoma in vitro and in vivo. *Eur J Pharmacol* 654, 17-25.

Jost C, Marin M and Kaelin WJ (1997) p73 is a simian [correction of human] p53-related protein that can induce apoptosis. *Nature* 389, 191-194.

Kaghad M, Bonnet H, Yang A, Creancier L, Biscan J, Valent A et al. (1997) Monoallelically expressed gene related to p53 at 1p36, a region frequently deleted in neuroblastoma and other human cancers. *Cell* 90, 809-819.

Kitching R, Li H, Wong M, Kanaganayakam S, Kahn H and Seth A (2003) Characterization of a novel human breast cancer associated gene (BCA3) encoding an alternatively spliced proline-rich protein. *Biochim Biophys Acta* 1625, 116-121.

Klanrit P, Flinterman M, Odell E, Melino G, Killick R, Norris J et al. (2008). Specific isoforms of p73 control the induction of cell death induced by the viral proteins, E1A or apoptin. *Cell Cycle* 7, 205-215.

Kornitzer D, Sharf R and Kleinberger T (2001) Adenovirus E4orf4 protein induces PP2A-dependent growth arrest in Saccharomyces cerevisiae and interacts with the anaphase-promoting complex/cyclosome. *J Cell Biol 154*, 331-344.

Kotadia S, Kao L, Comerford S, Jones R, Hammer R and Megraw T (2008) PP2A-dependent disruption of centrosome replication and cytoskeleton organization in Drosophila by SV40 small tumor antigen. *Oncogene* 27, 6334-6346.

Kourmouli N, Jeppesen P, Mahadevhaiah S, Burgoyne P, Wu R, Gilbert D et al. (2004) Heterochromatin and tri-methylated lysine 20 of histone H4 in animals. *J Cell Sci* 117, 2491-2501.

Kucharski T, Gamache I, Gjoerup O and Teodoro J (2011) DNA Damage Response Signaling Triggers Nuclear Localization of the Chicken Anemia Virus Protein Apoptin. *J Virol*, Epub ahead of print.

Landry M, Sicotte A, Champagne C and Lavoie J (2009) Regulation of cell death by recycling endosomes and golgi membrane dynamics via a pathway involving Src-family kinases, Cdc42 and Rab11a. *Mol Biol Cell* 20, 4091-4106.

Lanz H, Florea B, Noteborn MHM, Backendorf C (2012) Development and application of an in vitro apoptin kinase assay. Anal Biochem, in press

Leliveld S, Dame R, Mommaas M, Koerten H, Wyman C, Danen-van Oorschot A et al. (2003) Apoptin protein multimers form distinct higher-order nucleoprotein complexes with DNA. *Nucleic Acids Res* 31, 4805-4813.

Leliveld S, Dame R, Rohn J, Noteborn M and Abrahams J (2004) Apoptin's functional N- and C-termini independently bind DNA. *FEBS Lett* 557, 155-158.

Leliveld S, Zhang Y, Rohn J, Noteborn M and Abrahams J (2003) Apoptin induces tumor-specific apoptosis as a globular multimer. *J Biol Chem* 278, 9042-9051.

Leon D and Canaves J (2003) In silico study of breast cancer associated gene 3 using LION Target Engine and other tools. *Biotechniques 35*, 1222-1226, 1228, 1230-1231.

Leung T and Ngan H (2010) Interaction of TAp73 and breast cancer-associated gene 3 enhances the sensitivity of cervical cancer cells in response to irradiation-induced apoptosis. *Cancer Res* 70, 6486-6496.

Li Y, Dowbenko D and Lasky L (2002) AKT/PKB phosphorylation of p21Cip/WAF1 enhances protein stability of p21Cip/WAF1 and promotes cell survival. *J Biol Chem* 277, 11352–11361.

Liu X, Elojeimy S, El-Zawahry A, Holman D, Bielawska A, Bielawski J et al. (2006) Modulation of ceramide metabolism enhances viral protein apoptin's cytotoxicity in prostate cancer. *Mol Ther* 14, 637-646.

Liu X, Zeidan Y, Elojeimy S, Holman D, El-Zawahry A, Guo G et al. (2006) Involvement of sphingolipids in apoptin-induced cell killing. *Mol Ther* 14, 627–636.

Loyola A, Huang J, LeRoy G, Hu S, Wang Y, Donnelly R et al. (2003) Functional analysis of the subunits of the chromatin assembly factor RSF. *Mol Cell Biol* 23, 6759-6768.

Loyola A, LeRoy G, Wang Y and Reinberg D (2001) Reconstitution of recombinant chromatin establishes a requirement for histone-tail modifications during chromatin assembly and transcription. *Genes Dev* 15, 2837-2851.

Maddika S, Bay G, Kroczak T, Ande S, Maddika S, Wiechec E et al. (2007) Akt is transferred to the nucleus of cells treated with apoptin, and it participates in apoptin-induced cell death. *Cell Prolif* 40, 835–848.

Maddika S, Booy E, Johar D, Gibson S, Ghavami S and Los M (2005) Cancer-specific toxicity of apoptin is independent of death receptors but involves the loss of mitochondrial membrane potential and the release of mitochondrial cell-death mediators by a Nur77-dependent pathway. *J Cell Sci* 118, 4485–4493.

Maddika S, Panigrahi S, Wiechec E, Wesselborg S, Fischer U, Schulze-Osthoff K et al. (2009) Unscheduled Akt-triggered activation of cyclin-dependent kinase 2 as a key effector mechanism of apoptin's anticancer toxicity. *Mol Cell Biol* 29, 1235-1248.

Maddika S, Wiechec E, Ande S, Poon I, Fischer U, Wesselborg S et al. (2008) Interaction with PI3-kinase contributes to the cytotoxic activity of apoptin. *Oncogene* 27, 3060-3065.

Majumder P, Chattopadhyay B, Mazumder A, Das P and Bhattacharyya N (2006) Induction of apoptosis in cells expressing exogenous Hippi, a molecular partner of huntingtin-interacting protein Hip1. *Neurobiol Dis* 22, 242–256.

Majumder P, Chattopadhyay B, Sukanya S, Ray T, Banerjee M, Mukhopadhyay D et al. (2007) Interaction of HIPPI with putative promoter sequence of Caspase-1 *in vitro* and *in vivo*. *Biochem Biophys Res Commun* 353, 80-95.

Majumder P, Choudhury A, Banerjee M, Lahiri A and Bhattacharyya N (2007) Interactions of HIPPI, a molecular partner of Huntingtin interacting protein HIP1, with the specific motif present at the putative promoter sequence of the Caspase-1, Caspase-8 and Caspase-10 genes. *FEBS J* 274, 3886–3899.

Mao T, Hsu C, Yen M, Gilks B, Sheu J, Gabrielson E et al. (2006) Expression of Rsf-1, a chromatin-remodeling gene, in ovarian and breast carcinoma. *Hum Pathol* 37, 1169-1175.

Marte B and Downward J (1997) PKB/Akt: connecting phosphoinositide 3-kinase to cell survival and beyond. *Trends Biochem Sci* 22, 355-358.

Michiels F and Collard J (1999) Rho-like GTPases: Their role in cell adhesion and invasion. *Biochem Soc Symp* 65, 125-146.

Nakayama K, Nakayama N, Jinawath N, Salani R, Kurman R, Shih Ie M et al. (2007) Amplicon profiles in ovarian serous carcinomas. *Int J Cancer* 120, 2613–2617.

Noteborn M and Koch G (1995) Chicken anaemia virus infection: molecular basis of pathogenicity. *Avian Pathol* 24, 11-31.

Noteborn M, Todd D, Verschueren C, de Gauw H, Curran W, Veldkamp S et al. (1994) A single chicken anemia virus protein induces apoptosis. *J Virol 68*, 346-351.

Noteborn M, Zhang Y and van der Eb A (1998) Apoptin specifically causes apoptosis in tumor cells and after UV-treatment in untransformed cells from cancer-prone individuals: a review. *Mutat Res* 400, 447-455.

Novak R and Phillips A (2008) Adenoviral-mediated Rybp expression promotes tumor cell-specific apoptosis. *Cancer Gene Ther* 15, 713-722.

Pekarsky Y, Hallas C, Palamarchuk A, Koval A, Bullrich F, Hirata Y et al. (2001) Akt phosphorylates and regulates the orphan nuclear receptor Nur77. *Proc Natl Acad Sci U S A 98*, 3690–3694.

Peng D, Sun J, Wang Y, Tian J, Zhang Y, Noteborn M et al. (2007) Inhibition of hepatocarcinoma by systemic delivery of Apoptin gene via the hepatic asialoglycoprotein receptor. *Cancer Gene Ther* 14, 66-73.

Perpelescu M, Nozaki N, Obuse C, Yang H and Yoda K (2009) Active establishment of centromeric CENP-A chromatin by RSF complex. *J Cell Biol* 185, 397-407.

Peters M, Jackson D, Crabb B and Browning G (2002) Chicken anemia virus VP2 is a novel dual specificity protein phosphatase. *J Biol Chem* 277, 39566-39573.

Pietersen A, Erkeland S, Rutjes S, Philipsen S, Medema J and Noteborn M (2005) Continuous apoptin expression in transgenic mice does not interfere with lymphocyte development and proliferation. *J Med Mol Biol* 2, 321–330.

Pietersen A, van der Eb M, Rademaker H, van den Wollenberg D, Rabelink M, Kuppen P et al. (1999) Specific tumor-cell killing with adenovirus vectors containing the apoptin gene. *Gene Ther 6*, 882–892.

Pogribny I, Ross S, Tryndyak V, Pogribna M, Poirier L and Karpinets T (2006) Histone H3 lysine 9 and H4 lysine 20 trimethylation and the expression of Suv4-20h2 and Suv-39h1 histone methyltransferases in hepatocarcinogenesis induced by methyl deficiency in rats. *Carcinogenesis 27*, 1180–1186.

Poon I, Oro C, Dias M, Zhang J and Jans D (2005) A tumor cell-specific nuclear targeting signal within chicken anemia virus VP3/apoptin. *J Virol* 79, 1339-1341.

Poon I, Oro C Dias M, Zhang J and Jans D (2005b). Apoptin nuclear accumulation is modulated by a CRM1-recognized nuclear export signal that is active in normal but not in tumor cells. *Cancer Res* 65, 7059-7064.

Porrás A, Bennett J, Howe A, Tokos K, Bouck N, Henglein B et al. (1996) A novel simian virus 40 early-region domain mediates transactivation of the cyclin A promoter by small-t antigen and is required for transformation in small-t antigen-dependent assays. *J Virol* 70, 6902-6908.

Qian C and Zhou M (2006) SET domain protein lysine methyltransferases: Structure, specificity and catalysis. *Cell Mol Life Sci* 63, 2755-2763.

Qin H, Wang J, Liang Y, Taniguchi Y, Tanigaki K and Han H (2004) RING1 inhibits transactivation of RBP-J by Notch through interaction with LIM protein KyoT2. *Nucleic Acids Res* 32, 1492-1501.

Raisner M and Madhani D (2006) Patterning chromatin: from and function for H2A.Z variant nucleosomes. *Curr Opin Genet Dev 16*, 119–124.

Rangasamy D, Greaves K and Tremethick J (2004) RNA interference demonstrates a novel role for H2A.Z in chromosome segregation. *Nat Struct Mol Biol* 11, 650–655.

Robert A, Smadja-Lamère N, Landry M, Champagne C, Petrie R, Lamarche-Vane N et al. (2006) Adenovirus E4orf4 hijacks rho GTPase-dependent actin dynamics to kill cells: a role for endosome-associated actin assembly. *Mol Biol Cell* 17, 3329-3344.

Rohn J, Zhang Y-H, Aalbers R, Otto N, den Hertog J, Henriquez N et al. (2002) A tumor-specific kinase activity regulates the viral death protein apoptin. *J Biol Chem*, 277, 50820-50827.

Rouse J and Jackson S (2002) Interfaces between the detection, signaling, and repair of DNA damage. *Science* 297, 547-551.

Rubie H, Hartmann O, Michon J, Frappaz D, Coze C, Chastagner P et al. (1997) N-Myc gene amplification is a major prognostic factor in localized neuroblastoma: results of the French NBL 90 study. *J Clin Oncol* 15, 1171-1182.

Saffery R, Irvine D, Griffiths B, Kalitsis P and Choo K (2000). Components of the human spindle checkpoint control mechanism localize specifically to the active centromere on dicentric chromosomes. *Hum Genet* 107, 376-384.

Sanders S, Portoso M, Mata J, Bähler J, Allshire R and Kouzarides T (2004) Methylation of histone H4 lysine 20 controls recruitment of Crb2 to sites of DNA damage. *Cell* 119, 603-614.

Sarcinella E, Zuzarte C, Lau N, Draker R and Cheung P (2007) Monoubiquitylation of H2A.Z distinguishes its association with euchromatin or facultive heterochromatin. *Mol Cell Biol* 27, 6457–6468.

Sastri M, Barraclough D, Carmichael P and Taylor S (2005) A-kinase-interacting protein localizes protein kinase A in the nucleus. *Proc Natl Acad Sci U S A* 102, 349-354.

Sato M, Yao V, Arap W and Pasqualini R (2010) GRP78 signaling hub a receptor for targeted tumor therapy. *Adv Genet* 69, 97-114.

Sauane M, Su Z, Dash R, Liu X, Norris J, Sarkar D et al. (2010) Ceramide plays a prominent role in MDA-7/IL-24-induced cancer-specific apoptosis. *J Cell Physiol* 222, 546-555.

Sawa C, Yoshikawa T, Matsuda-Suzuki F, Delehouzee S, Goto M, Watanabe H et al. (2002) YEAF1/RYBP and YAF-2 are functionally distinct members of a cofactor family for the YY1 and E4TF1/hGABP transcription factors. *J Biol Chem 277*, 22484–22490.

Schlisio S, Halperin T, Vidal M and Nevins J (2002) Interaction of YY1 with E2Fs, mediated by RYBP, provides a mechanism for specificity of E2F function. *EMBO J* 21, 5775–5786.

Schotta G, Lachner M, Sarma K, Ebert A, Sengupta R, Reuter G et al. (2004) A silencing pathway to induce H3-K9 and H4-K20 trimethylation at constitutive heterochromatin. *Genes Dev* 18, 1251-1262.

Schotta G, Sengupta R, Kubicek S, Malin S, Kauer M, Callén E et al. (2008) A chromatin-wide transition to H4K20 monomethylation impairs genome

integrity and programmed DNA rearrangements in the mouse. *Genes Dev* 22, 2048-2061.

Schwab M (1998) Amplification of oncogenes in human cancer cells. *Bioessays* 20, 473–479.

Shamay M, Barak O, Doitsh G, Ben-Dor I and Shaul Y (2002) Hepatitis B virus pX interacts with HBXAP, a PHD finger protein to coactivate transcription. *J Biol Chem* 277, 9982-9988.

Shih Ie M, Sheu J, Santillan A, Nakayama K, Yen M, Bristow R et al. (2005) Amplification of a chromatin remodeling gene, Rsf-1/HBXAP, in ovarian carcinoma. *Proc Natl Acad Sci U S A*, 102, 14004-14009.

Siddiqui H, Fox S, Gunawardena R and Knudsen E (2007) Loss of RB compromises specific heterochromatin modifications and modulates HP1alpha dynamics. *J Cell Physiol* 211, 131-137.

Sims J, Houston S, Magazinnik T and Rice, J. (2006) A trans-tail histone code defined by monomethylated H4 Lys-20 and H3 Lys-9 demarcates distinct regions of silent chromatin. *J Biol Chem* 281, 12760-12766.

Skoczylas C, Henglein B and Rundell K (2005) PP2A-dependent transactivation of the cyclin A promoter by SV40 ST is mediated by a cell cycle-regulated E2F site. *Virology* 332, 596-601.

Song G, Ouyang G and Bao S (2005) The activation of Akt/PKB signaling pathway and cell survival. *J Cell Mol Med* 9, 59-71.

Stanton S, Blanck J, Locker J and Schreiber-Agus N (2007) Rybp interacts with Hippi and enhances Hippi-mediated apoptosis. *Apoptosis* 12, 2197-2206.

Sun G, Tong X, Dong Y, Mei Z and Sun Z (2002) Identification of a protein interacting with apoptin from human leucocyte cDNA library by using yeast two-hybrid screening. *Sheng Wu Hua Xue Yu Sheng Wu Wu Li Xue Bao* 34, 369–372.

Sun,J, Yan Y, Wang X, Liu X, Peng D, Wang M et al. (2009) PTD4-apoptin protein therapy inhibits tumor growth *in vivo*. *Int J Cancer*, 124, 2973-2981.

Sun L, Kong B, Sheng X, Sheu J and Shih I (2010) Dendritic cells transduced with Rsf-1/HBXAP gene generate specific cytotoxic T lymphocytes against ovarian cancer in vitro. *Biochem Biophys Res Commun* 394, 633-638.

Swaminathan J, Baxter E and Corces V (2005) The role of histone H2Av variant replacement and histone H4 acetylation in the establishment of Drosophila heterochromatin. *Genes Dev* 19, 65–76.

Teodoro J, Heilman D, Parker A and Green M (2004) The viral protein Apoptin associates with the anaphase-promoting complex to induce G2/M arrest and apoptosis in the absence of p53. *Genes Dev* 18, 1952-1957.

Trimarchi J, Fairchild B, Wen J and Lees J (2001) The E2F6 transcription factor is a component of the mammalian Bmi1-containing polycomb complex. *Proc Natl Acad Sci U S A* 98, 1519–1524.

Tryndyak V, Kovalchuk O and Pogribny I (2006) Loss of DNA methylation and histone H4 lysine 20 trimethylation in human breast cancer cells is associated with aberrant expression of DNA methyltransferase 1, Suv4-20h2 histone methyltransferase and methyl-binding proteins. *Cancer Biol Ther* 5, 65–70.

Tsuda H, Hirohashi S, Shimosato Y, Hirota T, Tsugane S, Yamamoto H et al. (1989) Correlation between long-term survival in breast cancer patients and amplification of two putative oncogene-coamplification units: hst-1/int-2 and c-erbB-2/ear-1. *Cancer Res* 49, 3104-3108.

Van Den Broeck A, Brambilla E, Moro-Sibilot D, Lantuejoul S, Brambilla C, Eymin B et al. (2008) Loss of histone H4K20 trimethylation occurs in preneoplasia and influences prognosis of non-small cell lung cancer. *Clin Cancer Res* 14, 7237-7245.

Vanhaesebroeck B and Waterfield M (1999) Signaling by distinct classes of phosphoinositide 3-kinases. *Exp Cell Res* 253, 239–254.

Vignali M, Hassan A, Neely K and Workman J (2000) ATP-dependent chromatin-remodeling complexes. *Mol Cell Biol* 20, 1899-1910.

Voelkel-Johnson C, Hannun Y and El-Zawahry A (2005) Resistance to TRAIL is associated with defects in ceramide signaling that can be overcome by exogenous C6-ceramide without requiring down-regulation of cellular FLICE inhibitory protein. *Mol Cancer Ther* 4, 1320-1327.

Yang H and Mizzen C (2009) The multiple facets of histone H4-lysine 20 methylation. *Biochem Cell Biol* 87, 151-161.

Yang H, Pesavento J, Starnes T, Cryderman D, Wallrath L, Kelleher N et al. (2008) Preferential dimethylation of histone H4 lysine 20 by Suv4-20. *J Biol Chem* 283, 12085-12092.

Yu K, Itokawa T, Zhu M, Syam S, Seth A and Insogna K (2007) Breast cancer-associated gene 3 (BCA3) is a novel Rac1-interacting protein. *J Bone Miner Res* 22, 628-637.

Zhang Y-H, Leliveld S, Kooistra K, Molenaar C, Rohn J, Tanke H et al. (2003) Recombinant apoptin multimers kill tumor cells but are nontoxic and epitope-shielded in a normal-cell-specific fashion. *Exp Cell Res* 289, 36-46.

Zhang Y, Kooistra K, Pietersen A, Rohn J and Noteborn M (2004) Activation of the tumor-specific death effector apoptin and its kinase by an N-terminal determinant of simian virus 40 large T antigen. *J Virol* 78, 9965-9976.

Zheng L, Schickling O, Peter M and Lenardo M (2001) The death effector domain-associated factor plays distinct regulatory roles in the nucleus and cytoplasm. *J Biol Chem 276*, 31945–31952 .

Zhou B and Elledge S (2000) The DNA damage response: putting checkpoints in perspective. *Nature* 408, 433-439.

Zhou X, Liao J, Meyerdierks A, Feng L, Naumovski L, Bottger E et al. (2000) Interferon-alpha induces nmi-IFP35 heterodimeric complex formation that is affected by the phosphorylation of IFP35. *J Biol Chem* 275, 21364-21371.

Zhuang S, Landegent J, Verschueren C, Falkenburg J, van Ormondt H, van der Eb, A et al. (1995) Apoptin, a protein encoded by chicken anemia virus, induces cell death in various human hematologic malignant cells *in vitro*. *Leukemia* 9, S118-120.

Zhuang S, Shvarts A, van Ormondt H, Jochemsen A, van der Eb A and Noteborn M (1995). Apoptin, a protein derived from chicken anemia virus, induces p53-independent apoptosis in human osteosarcoma cells. *Cancer Res* 55, 486-489.

Appendices

Summary

The human body consists of very many cells, in the order of trillions. Each of these trillions of cells has its own function, and together, groups of cells with similar functions make up the various organs of which we are comprised. Remarkably enough, all of these cells, as different as they are, – consider the cells making up the heart muscle, the ones in our brain, the ones lining our stomach and intestines, even the ones making our bones – all originate from one single cell: the fertilized oocyte. The fertilized oocyte lies at the very basis of life, and has the potential to replicate and specialize (i.e. differentiate) into each of the different types of cells required in the developing embryo. It does so according to a strictly regulated set of rules, that dictate which cells are to divide, when they are to do so, when they are to become more specialized, even which cells must die and when – e.g., to create the spaces between our fingers and toes. After birth, and throughout life, these rules continue to govern cellular replication, ensuring that old, damaged and/or dead cells are replaced, and wounds are healed, without creating any excess tissue or disturbing the functions of the different organs.

Cancer occurs as a violation of these rules. When the growth and death of a cell are no longer controlled, it can divide unlimitedly, and the resulting tumor could eventually invade and disrupt surrounding tissue, hindering organ function. If tumor cells manage to invade the blood stream, they could also travel to other sites in the body, where they can continue to replicate; this is termed metastasis. Standard therapy – surgery, radio- and/or chemotherapy, is not inherently selective for cancer cells. This results in lower efficiency and a host of unwanted side-effects, ranging from surgical amputation and life-threatening bone marrow depression, to the less dangerous but often equally upsetting loss of hair. Thus, in order to improve on efficiency

and minimize side-effects, more targeted therapies are needed. In order to design such therapies, we must understand how a cancer cell works. Otherwise stated, we must learn how it manages to extricate itself from the mechanisms that normally control cell division.

The hallmarks of a cancer cell

Research over the past several decades has shown that cancer cells share a number of features, which are characteristic of the process of tumorigenesis (**chapter 2**). First, cancer cells have the ability to produce their own growth factors, inducing proliferation independently of signals in their environment. Alternatively, they may stimulate surrounding cells (i.e., the cells in the tumor environment) to produce the growth factors for them. Second, cancer cells have found a way to circumvent the influence of growth inhibitory signals. Together with the first feature, this renders the cancer cell insensitive to extracellular control. This normally triggers the activation of internal controls, which program the cell to die. This type of cell death is termed apoptosis, and evasion of apoptosis is the third trait of malignant transformation. Yet cells still have one more method of intracellular control, which cancer cells must overcome in order to become truly immortal. With each round of cell division, the chromosomes, which carry the cell's DNA, become a little bit shorter. After a certain number of doublings, the chromosomes have become too short, and cell division is halted. Consequently, the fourth feature of carcinogenesis requires cancer cells to find a way to elongate the chromosome ends. Once cancer cells have acquired these first four traits, they are able to divide unlimitedly. In order to support this growth, they must develop the ability to create new vessels to ensure their blood supply (angiogenesis, the fifth cancer hallmark); the ability to invade these and other vessels and give rise to metastases is the sixth cancer hallmark. Cancer growth further requires a fundamental change in cellular metabolism (the seventh proposed hallmark), and,

importantly, evasion of destruction by the cells of the immune system (the eighth hallmark).

The acquisition of these cancer-typical traits is achieved by alterations in the cellular DNA, including mutations, deletions and amplifications. The majority of genes affected are those that encode the proteins involved in repairing damaged DNA. As a result, cancer cells accumulate errors in their DNA (a phenomenon termed genomic instability), which again enables them to acquire further cancer-typical features.

Apoptin and other proteins killing tumor cells

A number of proteins have recently been discovered to selectively kill cancer cells. That is, these proteins are able to distinguish between the normal and tumor cells, and kill the latter, while leaving the normal ones unharmed. This group of proteins killing tumor cells (PKTC) might therefore have the potential to be used to develop the ideal anti-cancer therapy.

The PKTC comprise proteins of various origins, including human proteins TRAIL, MDA7/IL24 and Par-4, the frog-derived Brevinin-2R, HAMLET, a complex between the milk protein alpha-lactalbumin and oleic acid (a component of human fat tissue, often used in detergents), and the viral proteins adenovirus E4ORF4, parvovirus NS1, and Chicken Anemia Virus-encoded protein apoptin.

While the PKTC are discussed in **chapter 3**, this thesis focuses on apoptin, which was the first of the PKTC to be discovered. Studies were carried out to gain more information on the mechanisms used by apoptin to sense oncogenic transformation and induce tumor-selective apoptosis. Knowledge on the proteins with which apoptin interacts within the human cell, and how these help apoptin to distinguish between normal and tumor cells, preventing its activation in normal

cells, and helping it to kill malignant cells, should aid in the design and development of novel, effective, selective, and hence more efficient therapies for cancer.

Apoptin interacts with FAM96B

In order to decipher the mechanisms behind apoptin's cellular activities and uncover new targets for anti-cancer therapies, we performed an analysis of apoptin-interacting proteins. One of the proteins identified in this way is the protein FAM96B (**chapter 4**). Identification of the FAM96B partners implicates a role of FAM96B in the regulation of the cell cycle, including the processes of sensing DNA damage and establishing sister chromatid cohesion. Through its interaction with FAM96B, apoptin is very likely also linked to these processes.

Apoptin & the nucleolus

One of the characteristic features of apoptin is its nuclear localization in tumor cells, versus its cytoplasmic localization in normal cells. Within the tumor cell nucleus, apoptin can be found in specialized regions, one of which is the nucleolus (**chapter 5**). The nucleolus is the cell's ribosome factory, which produces the ribosomes required to drive protein synthesis. Furthermore, the nucleolus has roles in coordinating the DNA damage response and inducing apoptosis. Apoptin is found to associate with nucleolar chromatin as well as various nucleolar proteins, suggesting that apoptin localizes to the nucleolus of tumor cells to shut off protein synthesis and induce apoptosis in response to DNA damage.

Apoptin interacts with BCA3 and is dephosphorylated by PP2A

Another characteristic feature of apoptin activity is its phosphorylation, which can be detected only in tumor cells, and is likely to be the trigger for apoptin nucle(ol)ar localization and apoptosis induction. In **chapter 6** we discuss the discovery of another

apoptin-interacting protein, BCA3. BCA3 led us to the involvement of PP2A B56δ in apoptin phosphorylation, while a second line of investigation led us to the involvement of PP2A B56γ in the same process. Dephosphorylation by the major tumor suppressor protein phosphatase 2A (PP2A) appears to fulfill the important function of keeping apoptin unphosphorylated, and, hence, inactive, in normal cells, while the loss of PP2A activity in tumor cells results in phosphorylation, and hence activation of apoptin.

Future perspectives

Apoptin is a small, avian-virus derived protein that has the remarkable ability to sense differences between normal and tumor cells, and is able to kill the latter while leaving the former unharmed. As discussed in **chapter 7**, novel insights into the mechanisms underlying this remarkable behavior should lead not only to an improved understanding of fundamental cellular biology, but also, importantly, to the development of new and improved anti-cancer strategies.

Samenvatting

Het menselijk lichaam bestaat uit triljoenen cellen. Elk van deze cellen heeft zijn eigen functie, en groepen van cellen met een vergelijkbare functie vormen samen de verschillende organen in het lichaam. Ondanks de grote verschillen tussen de verschillende celtypen – denk bijvoorbeeld aan de cellen die samen de hartspier vormen, de cellen in onze hersenen, die van onze darmen, en de cellen die onze botten vormen – ontstaan al deze cellen uit één en dezelfde cel: de bevruchte eicel. De bevruchte eicel is een zogenaamde 'stamcel', en bevat de unieke eigenschap dat hij zich kan vermenigvuldigen, maar dat sommige van de nieuw ontstane cellen zich ook kunnen specialiseren tot een bepaald type cel (oftewel, differentiëren). Op deze wijze ontstaat een volledig ontwikkeld embryo, met alle benodigde organen, uit deze eerste stamcel.

De deling en differentiatie van cellen is strikt gereguleerd, zodat elke cel weet of en wanneer het zich moet delen, wanneer het zich moet specialiseren en tot welk celtype, en zelfs wanneer het dood moet gaan, bijvoorbeeld om ruimte te creëren tussen de vingers en tenen. Ook na de geboorte, en zelfs gedurende het gehele leven, blijft deze regulatie van toepassing. Hierdoor kunnen oude, beschadigde, en/of dode cellen vervangen en wonden geheeld worden, zonder dat er ook maar één cel te veel of te weinig wordt aangemaakt, en weefselfunctie behouden blijft.

Kanker ontstaat juist wanneer deze regels geschonden worden. Wanneer de groei en dood van een cel niet meer gecontroleerd kunnen worden, kan deze cel zich blijven vermenigvuldigen. De zo ontstane tumor kan het omringende weefsel verstoren, waarbij de functie van het orgaan in gedrang kan komen. Tumorcellen kunnen bijvoorbeeld ook in de bloedbaan terechtkomen, en naar een ander deel van het

lichaam reizen, waar ze een nieuwe tumor kunnen vormen: metastasering.

De standaardbehandeling voor kanker bestaat uit chirurgie, gevolgd door radio- en/of chemotherapie, en is niet selectief voor kankercellen. Dit heeft een lagere therapie-efficiëntie tot gevolg, evenals een lange lijst aan ongewenste neveneffecten, variërend van chirurgische amputatie en levensbedreigende beenmergsuppressie, tot het minder gevaarlijke maar evenwel aangrijpende haaruitval. Om tot betere therapieën te komen is daarom een doelgerichtere aanpak vereist, waarbij gebruik wordt gemaakt van kennis van de eigenschappen van kankercellen.

De karakteristieke eigenschappen van een kankercel

Decennialang onderzoek heeft uitgewezen dat kankercellen een aantal karakteristieke eigenschappen bezitten (**hoofdstuk 2**). Ze kunnen ten eerste hun eigen groeifactoren produceren, waarmee ze hun eigen groei in stand kunnen houden, zonder daarvoor afhankelijk te zijn van hun omgeving. Andersom kunnen ze de cellen in hun omgeving ook zodanig beïnvloeden dat zij de benodigde groeistimulerende factoren uitscheiden. Kankercellen kunnen ten tweede signalen omzeilen, die anders voor inhibitie van de celgroei zouden zorgen. Deze twee eigenschappen zorgen dat kankercellen in principe ongevoelig worden voor controle van buitenaf. Echter, ze zijn dan nog wel gevoelig voor intracellulaire controlemechanismen. Een dergelijk mechanisme bestaat uit apoptose, een vorm van geprogrammeerde celdood, die wordt geactiveerd wanneer cellen zich ongecontroleerd delen. Het onderdrukken van apoptose vormt dan ook de derde typische eigenschap van kankercellen. De vierde eigenschap heeft te maken met de lengte van de chromosomen. Chromosomen bevatten het cellulaire DNA, en worden bij elke celdeling een stukje korter. Worden ze te kort, dan treedt er een mechanisme in werking waarbij de celdeling wordt stopgezet. Om door te kunnen groeien, zullen

kankercellen dus een manier moeten vinden om de uiteinden van de chromosomen weer te verlengen, bijvoorbeeld door het aanzetten van een bepaald eiwit, of door stukjes DNA aan elkaar te plakken en weer op andere plaatsen te knippen. Wanneer een kankercel deze vier eigenschappen bezit, is het in principe onsterfelijk geworden en kan het zich ongecontroleerd vermenigvuldigen. Hoe groter de tumor, hoe groter de behoefte aan zuurstof en voedingsstoffen; om hierin te kunnen voorzien zullen de kankercellen dus ook nieuwe bloedvaten aan moeten kunnen leggen: dit is de vijfde eigenschap. Komt een kankercel eenmaal in een bloed- of ander vat terecht, dan kan dit aanleiding geven tot metastasering (de zesde eigenschap). Om snel en veel te kunnen blijven groeien, ondergaan kankercellen bovendien een verandering in hun metabolisme (de voorgestelde zevende eigenschap); daarnaast zorgen kankercellen ervoor dat ze niet ontdekt en dus opgeruimd worden door cellen van het immuunsysteem.

Kankercellen verkrijgen de bovengenoemde typische eigenschappen door middel van veranderingen in hun DNA, waaronder mutaties, deleties en amplificaties. De genen die het vaakst door deze veranderingen worden getroffen, coderen vaak voor eiwitten die een rol spelen bij het herstel van DNA schade. Hierdoor kunnen genetische veranderingen zich ophopen (dit fenomeen wordt genetische instabiliteit genoemd), waardoor het weer makkelijker wordt voor de kankercel om de verdere benodigde eigenschappen te verkrijgen.

Apoptin en andere eiwitten die kankercellen doden

Recentelijk zijn er een aantal eiwitten ontdekt, waarvan bewezen is dat zij specifiek kankercellen doden. Deze eiwitten kunnen onderscheid maken tussen normale en tumorcellen, en doden de tumorcellen zonder schade aan te richten in de gezonde cellen. Deze groep eiwitten (in het Engels PKTC genoemd, voor “proteins killing tumor cells”) zouden dus de basis kunnen vormen voor de ontwikkeling van misschien wel hèt ideale medicijn tegen kanker!

De PKTC groep bestaat uit eiwitten van diverse origine, waaronder de menselijke eiwitten TRAIL, MDA7/IL24 en Par-4, het kikkereiwit Brevinin-2R, en HAMLET, een complex bestaande uit het melkeiwit alfa-lactalbumine en oliezuur, het meest voorkomende vetzuur in menselijk vetweefsel en een veelgebruikt bestanddeel in zeep. Daarnaast zijn er nog een aantal virale eiwitten, zoals E4ORF4, afkomstig van het adenovirus, NS1, afkomstig van het parvovirus, en apoptin, afkomstig van het Chicken Anemia Virus.

De verschillende PKTC zijn besproken in **hoofdstuk 3**, terwijl apoptin, die als eerste PKTC werd ontdekt, het hoofdonderwerp vormt van dit proefschrift. Er zijn experimenten uitgevoerd om informatie te verkrijgen over de mechanismen die door apoptin worden gebruikt om maligne transformatie te herkennen en tumorselectieve apoptose te induceren. Kennis over de cellulaire eiwitten waarmee apoptin een interactie aangaat, alsmede de manier waarop deze eiwitten bijdragen aan het onderscheid door apoptin tussen normale en tumorcellen, het aanzetten tot celdood in tumorcellen, en het voorkomen van activatie in normale cellen, zou belangrijke aanknopingspunten moeten opleveren voor de ontwikkeling van nieuwe, effectieve, selectieve en dus efficiëntere vormen van behandeling voor kanker.

Apoptin bindt aan FAM96B

Om aanknopingspunten te vinden voor het ontrafelen van de mechanismen achter het karakteristieke gedrag van apoptin en de ontwikkeling van nieuwe antikanker medicijnen, zijn we op zoek gegaan naar eiwitten die in de cel aan apoptin binden. Een van de eiwitten die we op deze manier gevonden hebben, was het tot dan toe nog relatief onbekende eiwit FAM96B (**hoofdstuk 4**). Analyses naar eiwitten die met FAM96B een interactie aangaan, hebben laten zien dat FAM96B betrokken is bij de regulatie van de celdeling, o.a. bij het herkennen van de aanwezigheid van genetische schade, en de verdeling van genetisch materiaal over de dochtercellen tijdens de

deling. Vanwege de associatie met FAM96B, lijkt het aannemelijk dat apoptin ook bij deze processen is betrokken.

Apoptin & de nucleolus

Een van de karakteristieke eigenschappen van apoptin betreft zijn kernlokalisatie in tumorcellen, in tegenstelling tot zijn lokalisatie in normale cellen, waar apoptin in het cytoplasma verblijft. Binnen de kern van de tumorcel houdt apoptin zich in specifieke gebieden op, waaronder de nucleolus (**hoofdstuk 5**). De nucleolus is de plek waar alle ribosomen worden gemaakt; deze zijn op hun beurt weer nodig zijn voor de eiwitsynthese in de cel. Daarnaast is het betrokken bij het coördineren van herstel van DNA schade en de inductie van apoptose. Apoptin bindt aan nucleolair chromatine (chromatine is het complex van DNA plus de eiwitten die ervoor zorgen dat het netjes in de celkern is opgevouwen) en wordt geassocieerd met verscheidene nucleolaire eiwitten, wat suggereert dat apoptin zich naar de nucleolus van kankercellen begeeft om eiwitsynthese (en dus celdeling) stop te zetten en apoptose te induceren in reactie op de aanwezigheid van DNA schade, hetgeen zoals eerder besproken veelvuldig voorkomt in tumorcellen.

Apoptin bindt aan BCA3 en wordt door PP2A gedefosforyleerd

Een andere typische eigenschap van apoptin, betreft zijn fosforylering: deze is alleen in kankercellen aan te tonen, en is waarschijnlijk de aanzet tot transport naar de kern/nucleolus en inductie van apoptose. **Hoofdstuk 6** beschrijft de identificatie van nog een interactiepartner van apoptin, namelijk BCA3. Via BCA3 en een ander, parallel lopend onderzoek, kwamen we op het spoor van het eiwit PP2A, welke een belangrijke rol lijkt te spelen in de fosforylering van apoptin. PP2A is een eiwit dat fosforylering kan verwijderen, en het lijkt erop dat het zorgt dat apoptin in de normale cel ongefosforyleerd blijft, terwijl in tumorcellen, waar PP2A ontbreekt, apoptin door toedoen van een nog onbekende kinase gefosforyleerd wordt en blijft.

Vooruitzichten voor de toekomst

Apoptin is een klein, vogelvirus eiwit dat de bijzondere eigenschap heeft dat het onderscheid kan maken tussen normale en tumorcellen, en de laatste categorie cellen kan doden, terwijl het de eerste categorie cellen ongemoeid laat. Zoals besproken in **hoofdstuk 7**, zouden nieuw opgedane inzichten in de werkingsmechanismen van dit bijzondere eiwit niet alleen moeten leiden tot een beter fundamenteel begrip van celbiologie, maar ook, zeer belangrijk, tot de ontwikkeling van nieuwe, betere, antikanker middelen.

Kompilashon di tésis

Tradusí ku yudansa di S.F. de Lima-Willems

E kurpa humano ta konsistí di miónes di sèl. Kada unu di e sèlnan tin su propio funshon i grupo di sèlnan ku funshonnan similar ta forma e diferente órganonan. Apesar di e gran diferensianan entre e diferente tipo di sèlnan, konsiderá por ehèmpel e sèlnan ku ta forma e múskulo di kurason, e sèlnan den nos serebro, esnan di nos intestino i e sèlnan ku ta forma nos wesunan, tur esakinan ta originá for di un solo sèl: e óvulo fekundá. E óvulo fekundá ta e asina yamá "sélula madre": e ta kontené e karakterístika úniko ku e por multipliká su mes, i tambe ku kada un di e sèlnan resien formá por spesialisá nan mes den un partikular tipo di sèl (mihó bisá diferensiá), asina ku e por yega na forma kada un di e tipo di sèlnan nesesario pa forma un ser humano.

E divishon i diferensiashon aki di e sèlnan ta estriktamente regulá, pa asina kada sèl sa si i ki ora e mester dividí, ki ora e mester spesialisá i den kua tipo di sèl, i asta ki ora e mester muri, por ehèmpel pa krea espasio entre nos dede i tenchinan. E regulashon aki ta keda na vigor despues di nasementu i asta durante henter nos bida. Dor di esaki sèlnan bieu, gastá, i/òf morto por ser remplasá i heridanan por kura, turestén sin ku ni sikiera un sèl di mas òf di ménos wòrdu formá, manteniendo e funshon di e tehido.

Kanser ta surgi presisamente ora e reglanan aki ser violá. Ora ku e kresementu i morto di un sèl no por ser kontrolá mas, e sèl aki por keda multipliká su mes. E tumor ku ta surgi por perhudiká e tehido rondó di dje, lokual tin komo konsekuensia ku e funshon di e órgano por ser afektá. Sèlnan di e tumor por drenta tambe den e sirkulashon

di sanger i biaha pa otro partinan di kurpa, kaminda nan por forma un tumor nobo, esta metástasis.

E tratamentu standardisá pa kanser ta konsistí di sirugia, siguí pa radio i/o chemoterapia, i no nesesariamente ta selektivo pa sèlnan maligno. Esaki tin komo konsekuensia un efisiensia abou di terapia, meskos ku un lista largu di efektonan sekundario indeseabel, ku ta varia for di amputashon kirúrgiko i depreshon di medula ku por ta mortal, te e esun ménos peligroso pero si mas konosí i sigur hopi konmovedor, ku ta kaida di kabei. Pa yega na terapianan mihó, ta nesesario un aserkamentu mas efikas, kaminda ta hasi uso di konosementu di karakterístikanan di sèlnan di kanser.

E kualidatnan karakterístiko di un sèl di kanser

Investigashon di desena di aña a saka na kla ku sèlnan di kanser ta poseé algun kualidatnan karakterístiko pa medio di kual nan ta manten'é nan mes kresementu, sin ku nan ta dependé den esei di nan ambiente. Kontrali na esaki nan por influensiá e sèlnan den nan ambiente tambe asina ku nan por ekspresá e faktornan di kresementu nesesario. Na di dos lugá, sèlnan kanseroso por aludí siñalnan ku di otro manera por a sòru pa inhibishon di e kresementu di sèlnan. E dos kualidatnan aki ta sòru ku sèlnan kanseroso en prinsipio ta bira insensibel pa kontròl di pafó. Sinembargo nan ta ketu bai sensibel p'e mekanismo di kontròl entre e sèlnan. Un mekanismo asina ta konsistí di apóptosis, un forma di morto selular programá ku ta ser aktivá ora sèlnan dividí fuera di kontròl. Pa e motibu ei, supreshon di apóptosis ta forma e di tres kualidat típiko di sèlnan kanseroso. E di kuater kualidat tin di haber ku e largura di e kromosómonan. Kromosómonan ta konten'é DNA di e sèl i ta bira mas kòrtiku kada bes ku esaki dividí. E momento ku nan bira muchu kòrtiku, un mekanismo ta drenta den akshon ku ta para e divishon di e sèl. Pa por sigui krese, e sèlnan kanseroso mester haña un manera pa alargá e puntanan di e kromosómonan, por ehèmpel dor di konvertí un

proteina determiná, òf dor di plak pida pida DNA na otro. E momentu ku un sèl di kanser ta poseé e kuater kualidatnan aki, en prinsipio el a bira inmortal i e por sigui multipliká su mes na un manera inkontrolabel. Mas grandi e tumor bira, mas grandi e nesesidat na oksígeno i nutrishon; pa por proporshoná den esaki, e sèlnan kanseroso tambe lo mester forma tubu di sanger nobo: esaki ta e di sinku kualidat. Si un sèl kanseroso resultá den un tubu di sanger òf kualkier otro tubu esaki por tin metastatis komo konsekuensia (e di seis kualidat). Pa por sigui krese rápidamente, sèlnan kanseroso ta pasa den un kambio di metabolismo (e di shete kualidat); banda di esei, sèlnan kanseroso ta sòru pa nan no ser deskubrí i pikí dor di e sèlnan di e sistema inmune.

Sèlnan di kanser ta atkerí tur e kualidatnan típiko aki pa medio di kambionan den nan DNA, manera mutashon, eliminashon, i amplifikashon. E gènnan ku mas ta ser afektá dor di e kambionan aki, ta kodifiká hopi bes pa proteinanan ku ta hunga un ròl den reparashon di DNA. Pa e motibu aki kambionan genétiko ta akumulá (e fenómeno aki ta ser yamá instabilidat genétiko), fasilitando e rekuperashon di e sobra kualidatnan nesesario pa yega na un estado maligno.

Apòptin i otro proteina ku ta mata sèlnan kanseroso

Resientemente a deskubrí algun proteina, di kual a proba ku nan ta mata sèlnan kanseroso spesífikamente. E proteinanan aki por distinguí entre sèlnan normal i esnan maligno, i ta mata e sèlnan kanseroso sin daña esnan salú. E proteinanan aki (yamá na ingles “PKTC”, esta “Proteins Killing Tumor Cells”) kisas por bai forma e base pa desaroyo di e medisina ideal kontra kanser!

E grupo di PKTC ta konsistí di proteina di diferente orígen, bou di kual e proteinanan humano TRAIL, MDA7/IL24 i Par-4, e proteina sapu Brevinin-2R, i HAMLET, un kompleho konsistiendo di e proteina

di lechi alfa-lactalbumina i ásido di zeta, e ásido di vèt ku mas ta paresé den tehido di vèt humano i ku a la bes ta un ingrediente hopi uzá den habon. Banda di esei tin un kantidat di proteina viral, manera E4ORF4, prosedente for di e vírus di adeno, NS1 prosedente for di e vírùs di parvo, i apòptin prosedente for di e vírus di anemia di galiña.

E diferente PKTC a ser tratá den **kapítulo 3**, miéntras ku apòptin, kual a ser deskubrí komo e promé PKTC, ta forma e tema prinsipal di e tésis aki. Eksperimentunan a ser kondusí pa haña informashon tokante di e mekanismonan ku apòptin ta uza pa rekonos'é transformashon maligno i introdusí apóptosis selektivo di e sèlnan tumorigeniko.

Konosementu di e diferente proteinanan selular ku kual apòptin ta drenta den interakshon, i tambe e manera ku e proteinanan aki ta aportá na e distinshon dor di apòptin entre sèlnan normal i esnan kanseroso, i e sistema pa aktivá apóptosis den esnan kanseroso, mientras evitando aktivashon den e sèlnan normal, mester bira e puntonan importante pa desaroyo di formanan di tratamentu di kanser nobo, kual lo ta mas efektivo, selektivo, i efisiente.

Apòptin ta uni su mes na FAM96B

Pa buska punto di salida pa esklaresimentu di e mekanismonan tras di komportashon karakterístiko di apòptin i e desaroyo di remedi nobo anti-kanseroso, nos a kuminsá buska proteina ku ta uni nan mes na apòptin den e sèl humano. Un di e proteinanan ku nos a haña na e manera aki ta FAM96B, kual ta un proteina ku te na e momento ei tabata relativamente deskonosí (**kapítulo 4**). Un análisis di e proteinanan ku ta den interakshon ku FAM96B a mustra ku esaki ta involukrá den regulashon di e divishon selular, inkluso e rekonosimentu di presensia di daño genétiko i distribushon di e kromosómonan durante e divishon. Debido na e asosashon ku

FAM96B, ta parse akseptabel ku apòptin tambe ta involukrá den e proseso aki.

Apòptin i e núkleo

Un di e kualidatnan karakterístiko di apòptin ta su lokalisashon den e núkleo di sèlnan di tumorigeniko, esaki kontrali na su lokalisashon den sèlnan normal, kaminda apòptin ta situá pafó di e núkleo, den e sitoplasma. Den e núkleo di e sèl maligno, apòptin ta ubiká den áreanan spesífiko por ehèmpel den e nukléulo. (**kapítulo 5**). E nukléulo ta e lugá kaminda tur ribosomanan ta ser formá: esakinan na nan turno ta nesesario pa e síntesis di proteinanan den e sèl. Ademas e nukléulo ta partisipá den e kordinashon di rekuperashon di daño den DNA i indukshon di apóptosis. A deskubri ku apòptin ta asosiá ku tantu kromatin komo ku vários proteina di e nukléulo, sugeriendo ku apòptin ta dirigí su mes pa nukléulo di sèlnan maligno pa para e síntesis di proteina i indusi apóptosis den reakshon riba presensia di daño di DNA, ku manera ya menshoná ta surgi frekuentamentu den e sèlnan kanseroso.

Apotin ta uni ku BCA3 anto ta ser dephoshorilatá dor di PP2A

Un otro kualidat típiko di apòptin ta e echo ku e por ser fosforilatá: esaki por ser demonstrá solamente den e sèlnan kanseroso, i probablemente e ta e empuhe pa transporte di apòptin pa e núkleo i nukléulo, i indukshon di apóptosis. **Kapitulo 6** ta dskribí e identifikashon di un otro partner di interakshon di apòptin, esta BCA3. Pa medio di BCA3 i un otro investigashon ku a kore paralelo, nos a bin kontra e proteina PP2A, ku a resultá di tin un papel prinsipal den e proseso di fosforilá apòptin. PP2A ta un proteina ku por kita fosforilashon, i ta parse ku e ta sòru pa apòptin keda no fosforilá den e sèl normal, miéntras ku den e sèlnan di tumor, kaminda ta karesé di PP2A, apòptin ta bira i keda fosforilá.

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Curriculum vitae

The author of this thesis was born July 31, 1982 on the Dutch Caribbean island of Curaçao, and raised in the fisherman's village of Boka Samí (St. Michael). Her native language is Papiamentu, and she is also fluent in Dutch, English, Spanish, French and Italian. After graduating from Radulphus College in 2000 with the highest honors, she moved to the Netherlands. Here, she studied Life Science & Technology at the Delft University of Technology and Leiden University, obtaining her Masters degree in 2005 with a double profile in Functional Genomics and Cell Diagnostics, with honors. During that time, she also earned her Unitech degree, having studied Business and Management at the Paris Institute of Technology (Ecole de l'INA PG), Paris, France, completed with an internship at Siemens Medical Health Services in Milan, Italy. She then started working on her PhD thesis with prof. Mathieu Noteborn, in his newly appointed Biological Chemistry group. Having completed her doctorate in Medicine (2006-2008), she left the lab in 2009 to carry out her clinical rotations. In August 2011, she obtained her Medical Degree with honors. Following the defense of her thesis, she plans to return to Curaçao, where she will combine her post-doctoral research with clinical residency at the Sint Elisabeth Hospital.

List of publications

Zimmerman RME, Sun J, Tian J, de Smit M, Danen-van Oorschot AA, Rotman M, Snel P, Voskamp P, Noteborn MHM, Backendorf C. Cellular partners of the apoptin-interacting protein 3. Submitted

Zimmerman RME, Florea BI, Backendorf C, Noteborn MHM. Apoptin interaction with chromatin. Manuscript in preparation.

Zimmerman RME*, Peng DJ*, Lanz HL, Zhang YH, Danen-van Oorschot AA, Qu S, Backendorf C, Noteborn MHM. PP2A inactivation is a crucial step in triggering apoptin-induced tumor-selective cell killing. Submitted.

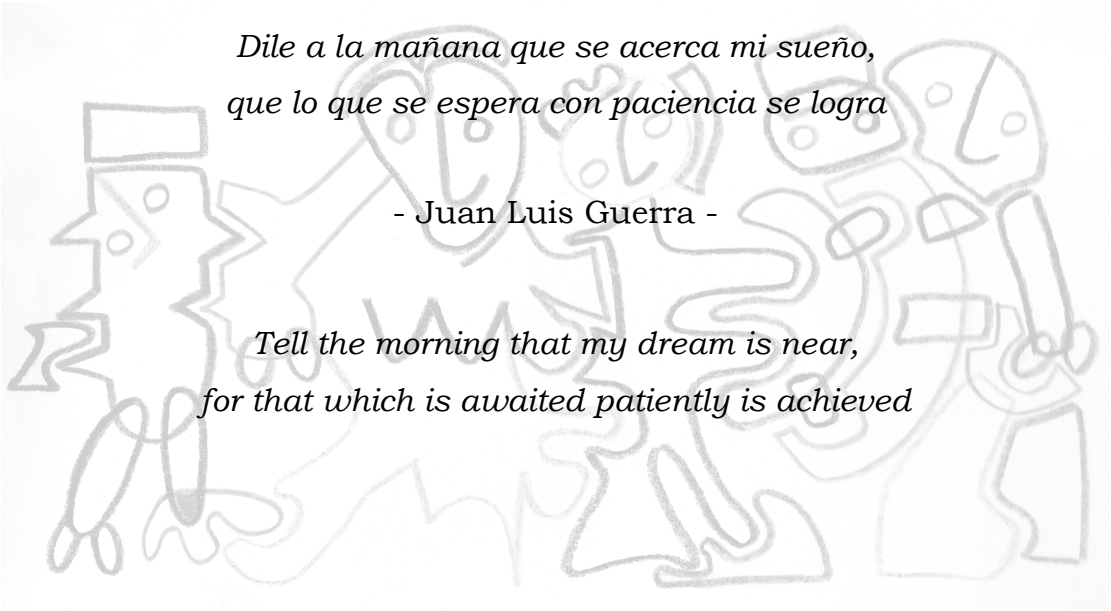
Bruno P, Brinkmann CR, Boulanger MC, Flinterman M, Klanrit P, Landry MC, Portsmouth D, Borst J, Tavassoli M, Noteborn M, Backendorf C, **Zimmerman RM**. Family at last: highlights of the first international meeting on proteins killing tumour cells. Cell Death Differ. 2009; 16:184-6.

Backendorf C, Visser AE, de Boer AG, **Zimmerman R**, Visser M, Voskamp P, Zhang YH, Noteborn M. Apoptin: therapeutic potential of an early sensor of carcinogenic transformation. Annu. Rev. Pharmacol. Toxicol. 2008;48:143-69.

Cleton-Jansen AM, van Eijk R, Lombaerts M, Schmidt MK, Van't Veer LJ, Philippo K, **Zimmerman RM**, Peterse JL, Smit VT, van Wezel T, Cornelisse CJ. ATBF1 and NQO1 as candidate targets for allelic loss at chromosome arm 16q in breast cancer: absence of somatic ATBF1 mutations and no role for the C609T NQO1 polymorphism.BMC Cancer. 2008;8:105.

Lombaerts M, van Wezel T, Philippo K, Dierssen JW, **Zimmerman RM**, Oosting J, van Eijk R, Eilers PH, van de Water B, Cornelisse CJ, Cleton-Jansen AM. E-cadherin transcriptional downregulation by promoter methylation but not mutation is related to epithelial-to-mesenchymal transition in breast cancer cell lines. Br. J. Cancer. 2006;94:661-71.

*contributed equally to the manuscript



Dile a la mañana que se acerca mi sueño,
que lo que se espera con paciencia se logra

- Juan Luis Guerra -

Tell the morning that my dream is near,
for that which is awaited patiently is achieved